IMMUNOLOGICAL STUDIES OF PSORIATIC ARTHRITIS

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Immunological studies of psoriatic arthritis

By

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ABSTRACT

Since little is known about the contribution of B cells to the pathology of psoriatic arthritis (PsA), this study investigated the possible recruitment of peripheral blood B cells to sites of inflammation by differential expression of chemokine receptors CXCR3, CXCR4, CXCR5, CCR1, CCR2, CCR5 and CCR6. Secondly, we explored the role of B cell activating factor (BAFF) in PsA. The chemokine receptor expression analysis did not reveal any significant differences (p-values >0.05 for all receptors) both at the RNA and the protein level when comparing PsA patients (n = 13) to rheumatoid arthritis (RA) patients (n = 15) and healthy controls (n = 10). Also, there was no significant difference (p = 0.1291) in plasma BAFF levels between PsA (n = 37) or RA patients (n = 24) and healthy controls (n = 35). Our study does not provide supporting evidence of B cell involvement in PsA, however it was limited to the analysis of peripheral blood.

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
APRIL	A proliferation-inducing agent
BAFF	B cell activating factor
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
DMARD	Disease-modifying antirheumatic drug
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tretraacetic acid
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GC	Germinal center
HEV	High endothelial venules
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
MTX	Methotrexate
NK cells	Natural killer cells
NSAID	Non-steroidal anti-inflammatory drug
OCP	Osteoclast precursor
OC	Osteoclast
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PsA	Psoriatic Arthritis
RT-PCR	Reverse transcription polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SLO	Secondary lymphoid organ
SLE	Systemic lupus erythematosus
TACI	Transmembrane activator and calcium ligand interactor
TNF	Tumor-necrosis factor

CHAPTER 1

INTRODUCTION

1.1 PSORIATIC ARTHRITIS

1.1.1 Defining psoriatic arthritis

Psoriatic arthritis (PsA) is an inflammatory arthritis that is associated with psoriasis. The association between psoriasis and arthritis was first described by Alibert in 1818 but not until 1964 was Psoriatic arthritis recognized as a distinct disease entity by the American Rheumatism Association (Tam and Geier 2004). Today PsA is considered a subtype of spondyloarthritis, a group of related rheumatic diseases, which includes for example ankylosing spondylitis, reactive arthritis or arthritis of inflammatory bowel disease. They share certain clinical features such as inflammatory spinal pain and they are associated with the HLA-B27 allele (Hochberg 2003). To distinguish PsA from related diseases, the classification criteria developed by Moll and Wright have been traditionally used. A patient must have psoriasis and an inflammatory form of arthritis while being negative for rheumatoid factor and must present with one of the following clinical subtypes: 1) Polyarticular, symmetric arthritis (rheumatoid arthritis-like), 2) Oligoarticular (<5 affected joints), asymmetric arthritis, 3) distal interphalangeal (DIP)[1] joint predominant, 4) spondylitis[2] predominant or 5) arthritis mutilans[3] (Moll and Wright 1973). The heterogeneous picture of PsA however makes it still difficult to diagnose and research the

[1] DIP joints are the joints on fingers and toes closest to the nail

[2] Spondylitis describes an inflammation of one or more vertebrae

[3] Arthritis mutilans is a severe form of arthritis with destruction of joint cartilages and bony surfaces leading to deformities on mainly hands and feet

disease. Therefore the CASPAR (**Clas**sification criteria for **P**soriatic **Ar**thritis) group has recently developed a new classification system. This study group is the result of an international collaboration and the new criteria were based on a large study including 30 clinics in 13 countries. The CASPAR criteria are summarized in Table 1.1. These new criteria for PsA have better specificity, but less sensitivity than other criteria developed and are therefore beneficial for use in clinical research (Taylor, Gladman et al. 2006).

Moreover, the CASPAR criteria reflect the specific features of spondyloarthritis that makes it differ from other forms of inflammatory arthritis such as RA. Dactylitis for example is common in PsA and occurs in about 30% to 40% of patients (Hochberg 2003). Another hallmark is the inflammation at the enthesis[4] that occurs in 20% to 40% of PsA patients. A further prominent feature is the destruction of the nail (nail dystrophy[5]). Nail dystrophy is associated with joint involvement and is more common in patients with PsA than in patients with uncomplicated psoriasis. There is no correlation however between the severity of the skin disease and joint scores, still 30% to 40% of patients experience coincidental flares of skin disease arthritis.

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[4] Enthesis is the location where the tendon inserts into the bone

[5] Nail dystrophy is the chronic destruction of the nail plate and includes here detachment of the nail from the nail bed, pitting of the nail and hyperkeratosis

Table 1.1: Summary of CASPAR criteria for PsA.

To meet the CASPAR criteria a patient must have inflammatory articular disease (joint, spine or entheseal) with ≥3 points from the following 5 categories:

1. Evidence of current psoriasis, a personal history of psoriasis, or a family history of psoriasis (current psoriasis is defined as psoriatic skin or scalp disease present today as judged by a rheumatologist or dermatologist[6]. A personal history of psoriasis is defined as a history of psoriasis that may be obtained from a patient, family physician, dermatologist, rheumatologist, or other qualified health care provider. A family history of psoriasis is defined as a history of psoriasis in a first- or second-degree relative according to patient report.).
2. Typical psoriatic nail dystrophy including onycholysis[7], pitting, and hyperkeratosis observed on current physical examination.
3. A negative test result for the presence of rheumatoid factor by any method except latex but preferably by enzyme-linked immunosorbent assay or nephelometry, according to the local laboratory reference range.
4. Either current dactylitis, defined as swelling of entire digit, or a history of dactylitis recorded by a rheumatologist.
5. Radiographic evidence of juxtaarticular new bone formation appearing as ill-defined ossifications[8] near joint margins (but excluding osteophyte[9] formation) on plain radiographs of the hand or foot.

From Taylor, Gladman et al. 2006.

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[6] Current psoriasis is assigned a score of 2; all other features are assigned a score of 1

[7] Onycholysis is a loosening of the exposed portion of the nail from the nail bed

[8] Ossification is the process of creating bone

[9] Osteophyte is a small bony outgrowth

1.1.2 Epidemiology

Even though some patients with psoriasis could have coincidental rheumatoid arthritis (RA) epidemiological evidence supports the concept of PsA as distinct disease entity (Tam and Geier 2004; Gladman, Antoni et al. 2005). The prevalence of psoriasis in the general population is 2% to 3% while it occurs in 7% of arthritis patients. PsA is prevalent in 6% to 42% of psoriasis patients whereas only 2% to 3% of the general population present with an inflammatory arthritis (Gladman, Antoni et al. 2005). This variance in prevalence estimates for PsA in psoriasis patients are most likely due to the heterogeneity of the disease and the lack of validated diagnostic criteria available at the time (Cassell and Kavanaugh 2005).

In contrast to RA, which has a female predominance, PsA affects men and women equally. The onset of disease occurs between 30 and 55 years of age. The majority of patients (70%) have psoriasis before developing PsA. In the remainder of patients the two conditions either occur at the same time (15%) or arthritis precedes the onset of PsA (15%) (Hochberg 2003).

1.1.3 Pathogenesis

The cause of PsA is not known but it is believed that genetic, immunological and environmental factors play a role. Additionally, a distinctive vascularity as well as bone remodeling contribute to the features seen in PsA pathology. It is similar to RA but there are unique features that set PsA apart as a distinct disease entity.

a) Genetics

There is compelling evidence that PsA has a strong genetic component. Actually 40% of PsA patients have a first-degree relative with either psoriasis or PsA (Cassell and Kavanaugh 2005; Turkiewicz and Moreland 2007). Several genetic susceptibility loci have been reported and the strongest association has been made with the major histocompatibility complex (MHC) region. Population studies have shown an increased frequency for the MHC class I alleles HLA-B27, HLA-B38, HLA-B39 and HLA-Cw6 in PsA patients. HLA-B27 is associated predominantly with spinal disease and the allele is typical of a spondyloarthritis, as mentioned earlier. On the other hand, alleles HLA-B38 and HLA-B39 are more frequent in patients with peripheral polyarthritis (Rahman and Elder 2005). The MHC class II allotype HLA-DR4 is also linked to peripheral arthritis in PsA patients (Cassell and Kavanaugh 2005). In psoriasis, the MHC class I alleles HLA-B13 and HLA-B17 have been linked to disease as well as HLA-Cw6 (Veale and FitzGerald 2002; Rahman and Elder 2005). In PsA patients, HLA-Cw6 is linked to an earlier age of onset for their psoriasis (Gladman, Cheung et al. 1999). The importance of the MHC region for PsA is further supplemented by linkage studies. A sibling pair analysis for PsA suggested higher HLA haplotype sharing among siblings that are both affected by PsA (Gladman, Farewell et al. 2003). And from the eight susceptibility loci for psoriasis (PSORS1-7 and PSORS9), the most important susceptibility locus PSORS1 falls within the MHC (Schon and Boehncke 2005).

Non-HLA genes within the MHC have been investigated as well. A TNF-α promoter polymorphism or a gene in linkage disequilibrium with TNF-α may contribute to

predisposition to psoriasis and PsA (Veale and FitzGerald 2002; Cassell and Kavanaugh 2005; Turkiewicz and Moreland 2007). The MHC class I chain –related A (MICA)-A9 polymorphism is another susceptibility factor in PsA (Gonzalez, Martinez-Borra et al. 2002). Outside the MHC region, IL-1 has been identified as a susceptibility locus in PsA (Rahman, Sun et al. 2006). Furthermore, the killer immunoglobulin-like receptor (KIR2DS1) gene, which has HLA-C as its ligand, showed an increased frequency in PsA patients (Williams, Meenagh et al. 2005). Interestingly, having an affected father increases the probability of disease in the children as there is excessive paternal transmission in PsA (Rahman, Gladman et al. 1999).

b) Immunology

The inflammatory nature of PsA is shown both in skin and joint. Psoriatic skin is characterized by epidermal hyperplasia[10], infiltration of lymphoid cells and abnormalities in vasculature (Veale and FitzGerald 2002). The same is true for the joint with synovial lining hyperplasia and infiltration of lymphocytic cells in the synovium and at sites of entheseal involvement (Veale and FitzGerald 2002; Gladman 2005). This is similar to what is seen in the inflamed synovium of rheumatoid arthritis (RA) but the inflammation in the PsA synovium exhibits less synovial lining hyperplasia, fewer macrophages and a higher vascularity compared to RA (Turkiewicz and Moreland 2007). Fig. 1.1 shows the histological appearance of normal skin compared to a psoriatic lesion.

[10] Hyperplasia is an abnormal increase in the number of cells

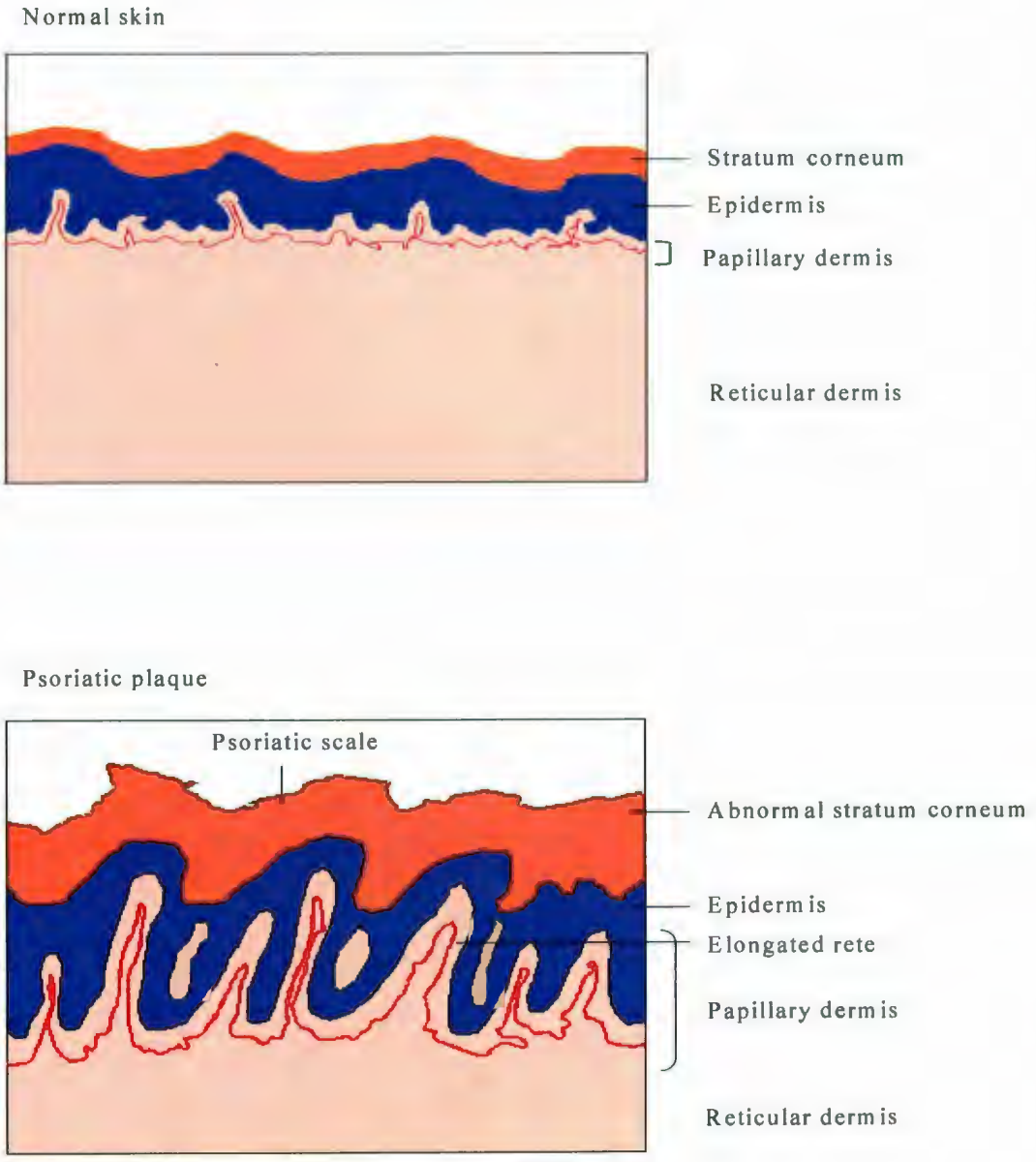


Figure 1.1: Skin histology diagrams in normal skin and psoriatic plaque.

The epidermal rete[11] become elongated and protrude into the dermis (Lowes, Bowcock et al. 2007). There is increased proliferation of keratinocytes in the epidermis but these cells are not completely differentiated and can therefore not stack normally. This abnormality of the stratum corneum[12] leads to the scales typically seen in psoriatic skin (Lowes, Bowcock et al. 2007). The blood vessels in the dermis are enlarged as well and stretch in between the epidermal rete.

Changes in vascularity are also seen in the PsA synovium (see 1.1.3 d) as well as thickening of the synovial membrane as shown in Figure 1.2. Leukocytes can enter the inflamed skin and joint as the activated endothelial cells express adhesion molecules to facilitate their transmigration. In the psoriatic skin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin enable leukocytes to enter the skin (Lowes, Bowcock et al. 2007). The same adhesion molecules are expressed by endothelial cells of the synovium yet E-selectin is expressed at lower levels (Veale, Ritchlin et al. 2005).

[11] Epidermal rete are downward undulations of the epidermis

[12] Stratum corneum is the outermost layer of the epidermis

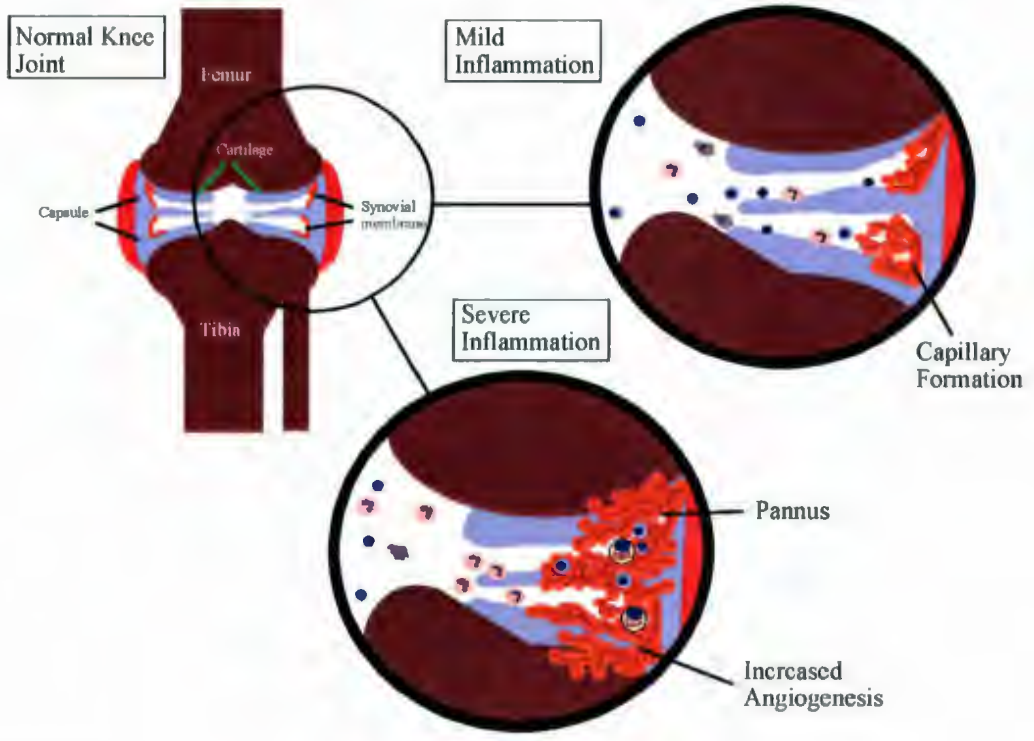


Figure 1.2: Normal joint features compared to joint with early and established inflammation. The established inflammation shows the pannus formation and the effects on the bone.

The infiltrating leukocytes in the synovium are mainly neutrophils, macrophages and T cells (Kruithof, Baeten et al. 2005). $CD4^+$ T cells are the most common lymphocyte in the synovium as well as the skin however $CD8^+$ T cells outnumber $CD4^+$ T cells at the enthesis and in the synovial fluid (Veale and FitzGerald 2002). The infiltrate in the skin is similar to the one in the synovium with neutrophils found in the stratum corneum and lymphocytes and monocytes infiltrating the dermis. T cells and dendritic cells are the most abundant lymphocytes in the dermis (Lowes, Bowcock et al. 2007).

In contrast to uncomplicated psoriasis, B cells are also part of the infiltrate in the skin (Veale, Barnes et al. 1994) and are commonly seen in the inflamed synovium (Turkiewicz and Moreland 2007). Their role in PsA is not clear, however they form germinal center like follicles as seen in ectopic lymphoid neogenesis (Canete, Santiago et al. 2007) and seem to be antigen-activated as determined by the analysis of immunoglobulin heavy chain variable (IgVH) genes (Gerhard, Krenn et al. 2002). So far, the focus has been on infiltrating T cells as $CD4^+$ T cells and $CD8^+$ T cells are clonally expanded in the skin and synovium. $CD8^+$ T cells seem to play a role in disease pathogenesis since they are the most common cell found in the synovial fluid and HLA class I is mainly associated with PsA (Veale and FitzGerald 2002). In the psoriatic skin, $CD8^+$ T cells are mainly found in the epidermis as they express the $\alpha e\beta 7$ integrin which binds to E-cadherin on keratinocytes (Krueger 2002; Lowes, Bowcock et al. 2007). $CD4^+$ T cells outnumber the $CD8^+$ T cells in the dermis similar to the ratio seen in peripheral blood.

Furthermore, different subsets of dendritic cells are found in the skin of psoriasis patients. Apart from the resident Langerhans cells there is an increase in dermal $CD11c^+$ dendritic cells (myeloid origin) in the lesion as well as mature dendritic cells as shown by CD83 or DC-LAMP expression (Krueger 2002). T cells and dendritic cells form aggregates, which most likely promote T cell activation since mature dendritic cells are potent T cell stimulators (Lowes, Bowcock et al. 2007).

The mediators of these inflammatory processes are cytokines. High levels of T_h1 cytokines TNF-α, IFN-γ and IL-2 have been detected in both synovium and skin (Veale and FitzGerald 2002; Turkiewicz and Moreland 2007). In the synovium, the T_h2 cytokine IL-10 is increased as well, but not IL-4 (Veale and FitzGerald 2002). Staining of PsA synovia additionally revealed expression of IL-1α, IL-1β and IL-15 (Veale and FitzGerald 2002). An important cytokine for disease pathogenesis is TNF-α. It is produced mainly by monocytes and macrophages but also by B cells, T cells, fibroblasts and keratinocytes (Choy and Panayi 2001; Mease 2006). TNF-α together with IL-1 activates synovial fibroblasts, osteoclasts[13] and chondrocytes[14] and additionally TNF-α promotes keratinocyte survival (Mease 2006). In turn, the activated fibroblasts produce other inflammatory cytokines such as IL-1, IL-6 and platelet-derived growth factors (Gladman 2005). TNF-α also activates endothelial cells, which in turn start expressing adhesion molecules to attract leukocytes to the site of inflammation as described above

[13] Osteoclast is a bone-resorbing cell

[14] Chondrocyte is a cartilage cell

(Choy and Panayi 2001). TNF-α and IL-1 are also responsible for joint damage (see 1.1.3e)).

They stimulate chondrocytes and synovial fibroblasts to release matrix metalloproteases that degrade connective-tissue matrix leading to cartilage erosion (Choy and Panayi 2001; Turkiewicz and Moreland 2007).

c) Environment

Both infection and trauma have been suggested to trigger PsA. Infection with human immunodeficiency virus has also been linked to psoriasis and PsA. A study from Zambia for example showed that 94% of PsA patients are HIV-positive and that the arthritis occurs in the early stages of infection (Veale and FitzGerald 2002; Hochberg 2003). Another study also reported an association between hepatitis C virus (HCV) and PsA as higher levels of antibodies for HCV were found in sera from PsA patients compared to healthy controls and psoriasis patients (Veale and FitzGerald 2002).

Physical trauma can also induce the onset of disease and this is especially known for psoriasis (Veale and FitzGerald 2002; Hochberg 2003; Turkiewicz and Moreland 2007). The Koebner phenomenon, which describes the development of psoriatic lesions at the site where the skin has been irritated by physical injury, has been described in a significant percentage of psoriasis patients. Another study reported that 24% of PsA patients suffered from a trauma to a joint before disease onset (Turkiewicz and Moreland 2007).

d) Vascularity

Vascular morphological changes can be seen in psoriatic skin lesions and PsA synovium (Veale, Yanni et al. 1993; Reece, Canete et al. 1999; Fraser, Fearon et al. 2001). They seem to be more important for the pathogenesis in PsA as studies by Veale state a more pronounced vascularity in PsA compared to RA (Veale and FitzGerald 2002). Elongated and tortuous vessels are characteristic for PsA synovium and skin which suggests a dysregulated angiogenesis resulting in new but immature vessels (Veale and FitzGerald 2002; Veale, Ritchlin et al. 2005). Angiogenic factors are most likely responsible for this increase in vascularity (Veale and FitzGerald 2002; van Kuijk, Reinders-Blankert et al. 2006). An increased expression of angiogenic growth factors such as transforming growth factor β (TGF-β), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) is found in psoriatic skin lesions. VEGF and TGF-β are also found at high levels in synovial fluid of patients with early PsA suggesting that angiogenesis is an early event in psoriasis and PsA (Veale, Ritchlin et al. 2005). Furthermore, another family of vascular growth factors called angiopoietins is found in perivascular areas of PsA joint synovium and co-localizes with VEGF expression (Veale and FitzGerald 2002). This increased expression of angiopoietins is supporting the role of growth factors as a cause for the vascular changes seen (Veale, Ritchlin et al. 2005). These similar findings of aberrant vascularity in both skin and joint may reflect a common neurovascular pathway (Veale and FitzGerald 2002).

e) Bone remodeling

The effects of the inflammation on the bone can be seen as bone erosions in affected joints. Radiographically the affected joints show loss in joint space, bone lesions, pencil-in-cup erosions (the bone becomes pointed and the adjacent joint looks cup-like due to erosions) and bone resorption (Ritchlin, Haas-Smith et al. 2003). Unlike in RA, new bone formation often accompanies the bone resorption, often in the same digit and at the site of the entheseal inflammation (Ritchlin, Haas-Smith et al. 2003; Walsh, Crotti et al. 2005). These findings point towards a disordered pattern of bone remodeling in PsA joints and furthermore these erosions differ from the bone loss seen in RA (Ritchlin, Haas-Smith et al. 2003). Altered signaling in osteoclast precursors is most likely responsible for the changes seen in PsA joints. Osteoclast precurors (OCP) are of monocyte/macrophage lineage and they can mature into osteoclasts, which degrade bone matrix and are the main cells responsible for bone resorption. In the circulation of PsA patients OCP are actually increased and studies in mice demonstrate that TNF-α can increase the number of circulating OCP (Ritchlin, Haas-Smith et al. 2003; Colucci, Brunetti et al. 2007). Fig. 1.3 shows the model proposed by Richtlin et *al.* (Ritchlin, Haas-Smith et al. 2003) for a possibly altered OCP signalling that leads to the changes seen in the joint. The end result in their model is a bi-directional bone resorption from the inside (subchondral) of the bone as well as from the inflamed outside of the bone (pannus-bone interface, see also Figure 1.2).

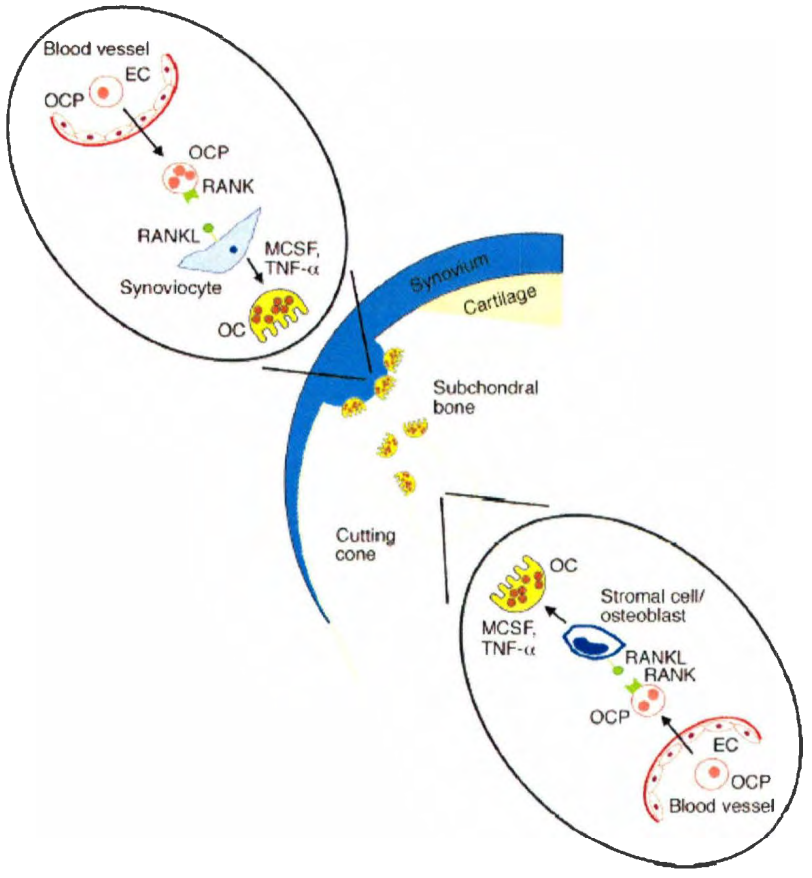


Figure 1.3: Schematic model of osteolysis in the psoriatic joint.

Circulating OCPs enter the synovium and become osteoclasts via RANKL signalling. OCPs in the subchondral bone undergo osteoclastogenesis as well.

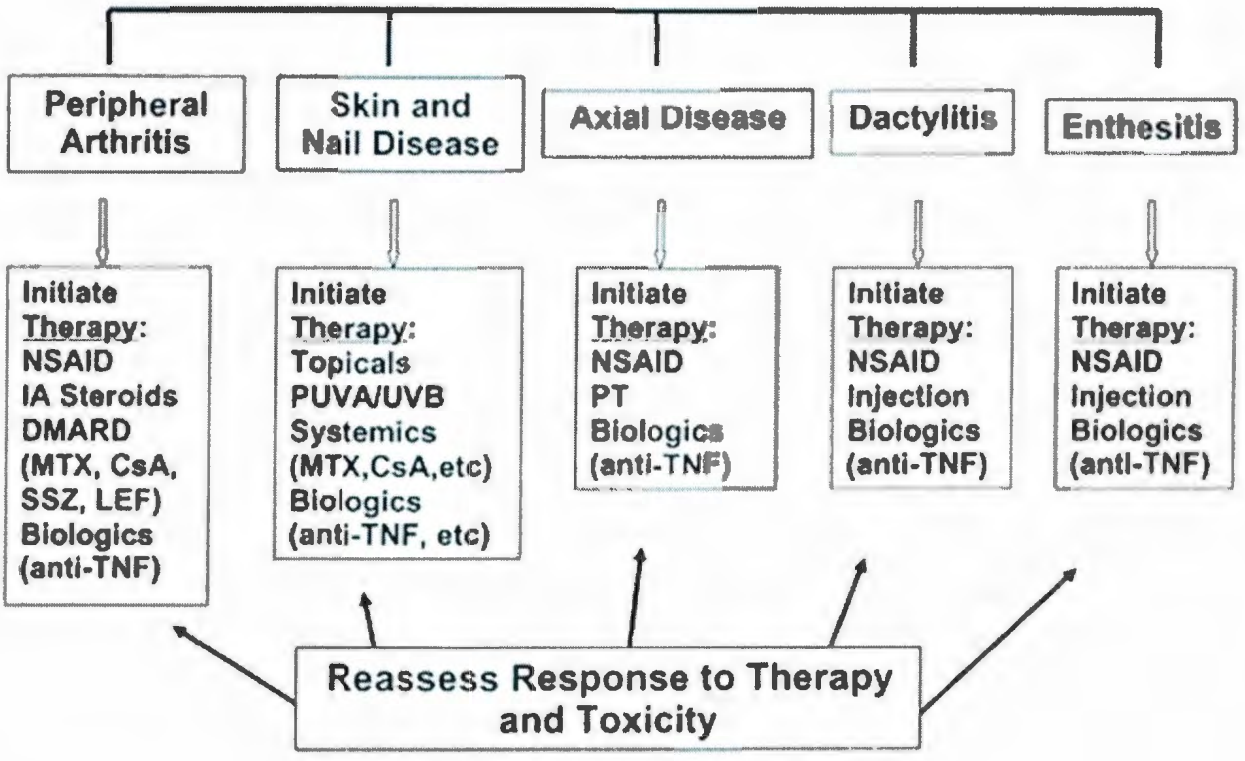
OC: osteoclasts; OCP: osteoclast precursor; RANKL: receptor activator of NF-κB ligand

From Ritchlin, Haas-Smith et al. 2003.

1.1.4 Treatment

Treatment for PsA needs to address both the skin and the joint involvement. Mild to moderate arthritis without evidence of progressive joint damage is usually treated with nonsteroidal anti-inflammatory drugs (NSAIDs) (Turkiewicz and Moreland 2007). Traditional NSAIDs like Ibuprofen and Diclofenac are unselective and block both cycloogxygenase (Cox) enzymes (Cox-1 and Cox-2), which are responsible for promoting inflammation and pain by producing prostaglandins. While they work well to reduce inflammation and improve pain and joint mobility, some patients experience considerable gastrointestinal side effects (Turkiewicz and Moreland 2007). New selective NSAIDs like Celecoxib specifically block the Cox-2 enzyme and this results in a better gastrointestinal tolerance. NSAIDs, however, do not modify the course of the arthritis and cannot prevent progressive joint disease (Gladman 2005). About 20% of PsA patients develop deforming and destructive arthritis (Turkiewicz and Moreland 2007) therefore needing more intensive treatment. Table 1.2 shows a treatment guideline for PsA patients according to the symptoms presented.

Table 1.2: Treatment guideline for PsA patients.



DMARD: Disease-modifying antirheumatic drug

MTX: Methotrexate

CsA: Cyclosporin A

SSZ: Sulfasalazine

LEF: Leflunomide

PUVA/UVB: Psoralen plus ultraviolet light A or B

Axial disease: Axial arthritis involving sacroiliac joint, spine or both

PT: Physical therapy

Adapted from Kavanaugh and Ritchlin 2006.

a) Traditional drugs

Methotrexate (MTX) is the standard disease-modifying antirheumatic drug (DMARD) used in treatment for PsA. It is an antimetabolite which inhibits cell proliferation. MTX is effective in ameliorating both the skin and the joint involvement, has a rapid mode of action and a good safety profile (Cassell and Kavanaugh 2005; Turkiewicz and Moreland 2007). Because MTX exhibits liver toxicity it is necessary to monitor liver and renal function during MTX treatment. If monitored every 6-8 weeks the risk of serious side effects can be avoided by stopping the drug when indicated (Hochberg 2003).

Other DMARDs used are sulfasalazine, cyclosporin A and leflunomide (see Table 1.2). Sulfasalazine is a sulfa drug which is effective for the treatment of peripheral arthritis in PsA by acting as an anti-inflammatory agent (Turkiewicz and Moreland 2007). Cyclosporin A, an immunosuppressive drug that blocks T cell proliferation, has renal toxicity and can cause hypertension therefore it is less commonly used or only in combination with other DMARDs (Hochberg 2003; Turkiewicz and Moreland 2007). Leflunomide inhibits the *de novo* synthesis of pyrimidines, which T cells need to proliferate, thus it hinders T cell activation and proliferation (Gladman 2005). As T cells play a role in PsA disease pathogenesis leflunomide seems effective in treatment of PsA. However, its disease modifying ability remains to be determined (Gladman 2005).

b) Biological response modifiers

With a greater understanding of disease pathology and the need for improved treatments, novel therapeutic options are being explored. Biological response modifiers are now used

for treatment of PsA after being proven effective in other autoimmune diseases such as RA. The most experience so far has been obtained with TNF-α inhibitors (Cassell and Kavanaugh 2005). Since the start of biologic drug development in the 1990s there are three TNF-α inhibitors currently available: etanercept (brand name Enbrel), infliximab (brand name Remicade) and adalimumab (brand name Humira) (Turkiewicz and Moreland 2007). They all exhibit their inactivating function by binding to TNF.

Etanercept. Etanercept is a recombinant human soluble TNF receptor. It is a fusion protein consisting of the extracellular portion of the human p75 TNF receptor linked to the Fc portion of human IgG1 (Gladman 2005). The US Food and Drug Administration (FDA) approved etanercept for the treatment of PsA in 2002. In a phase II and a phase III clinical trial, etanercept showed sustained efficacy in improving joint and skin disease and this was independent of background MTX treatment (this was also seen for other TNF inhibitors) (Turkiewicz and Moreland 2007). Thus etanercept enables patients who have longstanding disease and are on a DMARD regimen to decrease or discontinue their concomitant treatment. Etanercept also reduced progressive joint damage as assessed by radiographs (Cassell and Kavanaugh 2005). Etanercept has a good safety profile and very good tolerability with the most common side effect being adverse reactions at the injection site (Gladman 2005).

Infliximab. Infliximab is a chimeric monoclonal antibody that binds specifically to soluble and membrane-bound TNF-α. It consists of human constant regions and murine variable regions (Gladman 2005; Turkiewicz and Moreland 2007). Infliximab was assessed in several trials and showed good efficacy for improvement of joint

inflammation and skin disease (Cassell and Kavanaugh 2005; Gladman 2005; Turkiewicz and Moreland 2007).

Adalimumab. Adalimumab is a humanized anti-TNF-α monoclonal antibody. It is effective for treatment of both skin and joint manifestations and it also inhibits radiographic disease progression (Cassell and Kavanaugh 2005; Turkiewicz and Moreland 2007). Improvement in the quality of life for the adalimumab users was seen during the clinicals trials as well (Turkiewicz and Moreland 2007). The safety profile of adalimumab was consistent with previous results from RA trials (Gladman 2005; Turkiewicz and Moreland 2007). Since adalimumab is a humanized antibody the development of anti-chimeric antibodies by the patients is less likely (Gladman 2005).

Despite the success with TNF-α inhibitors, about 20% of PsA patients are refractory to the treatment (Cassell and Kavanaugh 2005).

1.2 Chemokines and their receptors

Chemokines, their receptors and their biological functions have been extensively reviewed (Luster 1998; Moser and Loetscher 2001; Le, Zhou et al. 2004; Esche, Stellato et al. 2005; Stein and Nombela-Arrieta 2005). In PsA, inflammatory chemokines have been detected in the inflamed synovium and synovial fluid and seem to help the recruitment of T cells to the site. The role of chemokines and their receptors in PsA is described in more detail in the Introduction of Chapter 3 (see p. 55).

1.2.1 Chemokine structure

Chemokines are small (6-14 kD) basic proteins that share 20 to 70 percent homology in their amino acid sequence. The superfamily of chemokines consists of more than 50 chemokines and has been subclassified on the basis of the position of their cysteine residues at the N-terminus. As shown in Table 1.3 there are four chemokine families, designated as C, CC (formerly β-chemokines), CXC (formerly α-chemokines) and CX3C, with C representing the number of cysteine residues at the N-terminus and X denoting the number of amino acids in between the first two cysteines (Luster 1998; Le, Zhou et al. 2004). The biggest family features the CC chemokines with 28 members (CCL1-CCL28) followed by the CXC chemokines with 16 members (CXCL1-CXCL16). The C chemokines and CX3C chemokines are the smallest families with only two or one member respectively. The CXC family of chemokines is further subdivided into those that have a glutamic acid-leucine-arginine (ELR^+) motif before the CXC sequence and those that do not (ELR^-) (Luster 1998; Le, Zhou et al. 2004). Generally, different chemokine families act on different leukocytes. In this way, ELR^+ CXC chemokines generally attract neutrophils (for example CXCL8, formerly IL-8) whereas ELR^- CXC chemokines act on lymphocytes (for example CXCL12, formerly SDF-1). The CC chemokines attract monocytes, eosinophils, basophils and lymphocytes (Luster 1998). C chemokines attract T cells while CX3C chemokine (fractalkine) is chemotactic for T cells, natural killer cells and monocytes. Based on their function, chemokines can be divided into homeostatic or constitutively expressed chemokines and inflammatory or

inducible chemokines. Homeostatic chemokines are involved in leukocyte development and trafficking and help maintain the structure and organization of lymphoid organs. Inflammatory chemokines are induced by proinflammatory signals and are important for both innate and adaptive immune responses, as they are able to activate and recruit effector leukocytes to sites of inflammation (Le, Zhou et al. 2004; Esche, Stellato et al. 2005).

Table 1.3: Chemokine families.

Chemokines	-ELR-	H/I	Synonyms	Major target cells showing chemotaxis
CC chemokines				
CCL1	NA	I	I-309, TCA3, P500	monocytes, T cells
CCL2	NA	I	MCP-1, MCAF (mouse; JE)	monocytes, T cells, basophils, NK cells, progenitors
CCL3	NA	I	LD78α, LD78β, MIP-1α	monocytes, T cells, NK cells, basophils, eosinophils, dendritic cells, hematopoietic progenitors
CCL4	NA	I	Act-2, G-26, HC21, H400, MIP-1β, LAG-1, SISγ, MAD-5	monocytes, T cells, dendritic cells, NK cells, progenitors
CCL5	NA	I	RANTES	T cells, eosinophils, basophils, NK cells, dendritic cells
CCL6	NA	I	C10 (mouse), MRP-1 (mouse)	macrophages
CCL7	NA	I	MCP-3	monocytes, T cells, eosinophils, basophils, NK cells, dendritic cells
CCL8	NA	I	MCP-2, HC14	monocytes, T cells, eosinophils, basophils, NK cells
CCL9	NA	I	MRP-2 (mouse), MIP-1γ (mouse)	T cells
CCL10	NA	I	CCF18	T cells
CCL11	NA	I	eotaxin	eosinophils, T cells
CCL12	NA	I	MCP-5 (mouse)	monocytes, T cells, eosinophils
CCL13	NA	I	MCP-4, NCC-1, CKβ10	monocytes, T cells, eosinophils
CCL14	NA	I	HCC-1, HCC-3, NCC-2, CKβ1, MCIF	monocytes, hematopoietic progenitors
CCL15	NA	I	HCC-2, NCC-3, MIP-5, Lkn-1, MIP-1	monocytes, T cells, eosinophils
CCL16	NA	I	NCC-4, LEC, HCC-4, LMC, LCC-1,CKβ12	T cells, neutrophils
CCL17	NA	H	TARC	T cells
CCL18	NA	H?	DC-CK1, PARC, MIP-4, CKβ7, DCCK1	naïve T cells
CCL19	NA	H	ELC, MIP-3β, exodus-3, CKβ11	T cells, B cells, dendritic cells, activated NK cells
CCL20	NA	H	MIP-3, LARC, exodus-1, ST38, CKβ4	T cells, B cells
CCL21	NA	H	SLC, 6Ckine, exodus-2, TCA4, CKβ9	T cells, B cells, dendritic cells, activated NK cells, macrophage progenitors
CCL22	NA	H	MDC, STCP-1, DC/B-CK	T cells, eosinophils
CCL23	NA	I	MIP-3, MPIF-1, CKβ8	dendritic cells, osteoclasts
CCL24	NA	I	MPIF-2, CKβ6, eotaxin-2	effector Th2 cells
CCL25	NA	H	TECK, CK15	memory T cells, B cells, immature thymocytes
CCL26	NA	I	eotaxin-3, IMAC, MIP-4α, TSC-1	eosinophils, T cells
CCL27	NA	H	ALP, skinkine, ILC, ESkine, PESKY, CTAK	CLA^+ T cells
CCL28	NA	H	MEC, CCK1	T cells
CXC chemokines				
CXCL1	ELR+	I	GROα, MGSA-α, NAP-3 (mouse/rat, KC, MIP-2, CINC-2β)	neutrophils, endothelial cells
CXCL2	ELR+	I	GROα, MIP-2α, MGSA-β, CINC-2α	neutrophils, endothelial cells
CXCL3	ELR+	I	GROγ, MIP-2α, CINC-2β	neutrophils
CXCL4	ELR-	I	PF4	fibroblasts, endothelial cells
CXCL5	ELR+	I	ENA-78	neutrophils
CXCL6	ELR+	I	GCP-2	neutrophils
CXCL7	ELR+	I	CTAPIII, NAP-2, LA-PF4, MDGF, LDGF, β-TG	fibroblasts
CXCL8	ELR+	I	IL-8, NAP-1	neutrophils, T cells, basophils, endothelial cells
CXCL9	ELR-	I	Mig	T cells, progenitors
CXCL10	ELR-	I	IP-10	T cells
CXCL11	ELR-	I	I-TAC	T cells
CXCL12	ELR-	H	SDF-1, SDF-1, PBSF	monocytes, B cells, hematopoietic progenitors, non-hematopoietic cells
CXCL13	ELR-	H	BLC, BCA-1	B cells
CXCL14	ELR-	I	BRAK, bolekine, MIP-2, BMAC, KS1	neutrophils, NK cells, B cells?
CXCL15	ELR-	H	lungkine	airspace neutrophils
CXCL16	ELR-	?	SR-PSOX, SEXCKINE	dendritic cells
C chemokines				
XCL1	NA	I	lymphotactin, SCM-1, ATAC	B cells, T cells, NK cells, neutrophils
XCL2	NA	I	SCM-1	B cells, T cells, NK cells, neutrophils
CX3C chemokine				
CX3CL1	NA	I	fractalkine, neurotactin	effector T cells

H, homeostatic chemokine; I, inflammatory chemokine; NA, not applicable. For definitions of the various synonyms, see Ref 21.

From Le, Zhou, et al. 2004.

1.2.2 Chemokine receptor families

Chemokines exert their function via chemokine receptors: there are eleven CC receptors (CCR1-11), six CXC receptors (CXCR1-6), one C receptor (XCR1) and one CX3C receptor (CX3CR1) as shown in Table 1.4 (Le, Zhou et al. 2004). CC receptors usually bind CC chemokines and CXC receptors bind CXC chemokines. There are known exceptions though, for example CXCL9, CXCL10 and CXCL11 can bind to CCR3 but serve as antagonists (Esche, Stellato et al. 2005). Also, there is redundancy in the chemokine system as some chemokine receptors can bind more than one chemokine and some chemokines bind to more than one receptor. The latter is true mainly for inflammatory chemokines whereas homeostatic chemokines usually bind to just one receptor. Additionally, chemokines can bind to two decoy chemokine receptors that have no signaling capability: Duffy antigen receptor for chemokines (DARC) and D6 (Esche, Stellato et al. 2005). CC as well as CXC chemokines can bind to these decoy receptors but they do not induce a calcium influx (Luster 1998).

Table 1.4: Chemokine receptor families.

Receptors	Synonyms	Chemokine ligands	Receptor-expressing cells
CCR			
CCR1	CKR1, CC CKR1, CMKBR1	CCL3,5,7,8,13,14,15,16,23	monocytes, immature DCs, T cells, PMNs, eosinophils, mesangial cells, platelets
CCR2	CKR2, CC CKR2, CMKBR2	CCL2,7,8,12,13	monocytes, immature DCs, basophils, PMNs, T cells, NK cells, endothelial cells, fibroblasts
CCR3	CKR3, CC CKR3, Eot R, CMKBR3	CCL5,7,8,11,13,14,15,24,26	eosinophils, basophils, T cells (Th2>Th1), DCs, platelets, mast cells
CCR4	CKR4, CC CKR4, CMKBR4, K5-5	CCL17,22	immature DCs, basophils, T cells (Th2>Th1), platelets
CCR5	CKR5, CC CKR5, ChemR13, CMKBR5	CCL3,4,5,8,11,13,14,20	Th1 cells, immature DCs, monocytes, NK cells, thymocytes
CCR6	GPR-CY4, CKR-L3, STRL22, CRY-6, DCR2, CMKBR6	CCL20	immature DCs, T cells, B cells
CCR7	BLR-2, CMKBR7	CCL19,21	mature DCs, T cells, B cells
CCR8	TER1, CKR-L1, GPR-CY6, ChemR1, CMKBR8	CCL1,4,16	monocytes, B cells, T cells, thymocytes
CCR9	GPR9-6	CCL25	T cells, thymocytes, DCs, macrophages
CCR10	GPR2	CCL27,28	T cells, melanocytes, dermal endothelia, dermal fibroblasts, Langerhans cells
CCR11	PPR1	CCL2,8,13,19,21,25	astrocytes
CXCR			
CXCR1	IL-8RA, IL-8R-I, IL-8R	CXCL2,3,5,6,7,8	PMNs, monocytes, astrocytes, endothelia, mast cells
CXCR2	IL-8RB, IL-8R-II, IL-8R	CXCL1,2,3,5,6,7,8	PMNs, monocytes, eosinophils, endothelia, mast cells
CXCR3	IP10/MigR, GPR9	CXCL9,10,11	T cells (Th1>Th2), B cells, NK cells, mesangial cells, smooth muscle cells, endothelia
CXCR4	HUMSTSR fusin, LESTR, HM89	CXCL12	hematopoietic progenitors, T cells, immature DCs, monocytes, B cells, PMNs, platelets, astrocyte, endothelia
CXCR5	BLR-1, MDR15	CXCL13	T cells, B cells, astrocytes
CXCR6	Bonzo, STRL33, TYMSTR	CXCL16	memory T cells
XCR			
XCR1	GPR5	XCL1, XCL2	T cells
CX3CR			
CX3CR1	GPR13, V28, CMKBRL1	CX3CL1	PMNs, monocytes, NK cells, T cells, astrocytes
Duffy	DARC	CXCL1,7,8, CCL1,5	red blood cells, endothelia
D6	CCR9,10, JAB61	CCL2,4,5,8,13,14,15	B cells

NK cell, natural killer cell; DC, dendritic cell; PMN, polymorphonuclear granulocyte

From Le, Zhou, et al. 2004.

1.2.3 Chemokine receptor signaling

Chemokine receptors are G-protein coupled receptors that have seven helical transmembrane domains that are connected by extramembranous loops (Allen, Crown et al. 2007). The NH_2-terminus and three extracellular loops are located outside the cell whereas the cytoplasmic COOH-terminus and three intracellular loops face towards the inside of the cell (Allen, Crown et al. 2007). These intracellular loops contain conserved serine and threonine residues that become phosphorylated upon receptor binding (Ono, Nakamura et al. 2003). Associated with the receptor is a heterotrimeric G protein, which consists of three subunits ($\alpha\beta\gamma$) and is responsible for receptor signaling. The Gα subunit interacts with the intracellular loops and is associated with the Gβ subunit which in turn interacts with the Gγ subunit (Allen, Crown et al. 2007). In the inactive state of the receptor, the Gα subunit binds GDP and is not bound to the intracellular loops of the receptor (Janeway 2001). When a ligand binds to the chemokine receptor the conformation of the receptor changes, which allows the G-protein to bind to the intracellular loops and the GDP of the inactive form is replaced by GTP. Upon GTP binding, the G protein dissociates into the Gα subunit and the G$\beta\gamma$ subunit. Both complexes activate downstream effectors that in turn activate signaling pathways that lead to the physiological response (Allen, Crown et al. 2007). Once the Gα subunit has bound to its effector, the GTPase activity of the Gα subunit is activated and the GTP is cleaved to GDP, which allows the subunit to reassociate with the G$\beta\gamma$ subunit (Janeway 2001).

1.2.4 Chemokine and receptor functions

Chemokines and their receptors have a variety of functions. One major function is their ability to direct and attract leukocytes and that in turn is important during leukocyte development, homeostasis and inflammation. The main focus of the following paragraphs will be on lymphocytes.

Lymphocyte development. Chemokines guide precursor cells to the right anatomical sites within primary lymphoid organs to ensure their proper development (Le, Zhou et al. 2004). Studies by Ma et *al.* showed that CXCR4 and its ligand CXCL12 are vital for B cell development (Ma, Jones et al. 1998). This is most likely due to the fact that this chemokine and receptor pair is needed to retain precursor cells within the fetal liver and the bone marrow (Ma, Jones et al. 1999). Chemokines are also important for T cell development in the thymus. CCR7 and its ligand CCL21 are important for colonization of the thymus with prethymocytes during embryogenesis (Liu, Ueno et al. 2005). CCR7 together with CCR9 and CXCR4 also help thymocytes to migrate to the different zones of the thymus during their development (Stein and Nombela-Arrieta 2005).

Lymphocyte homing. Chemokines control lymphocyte trafficking within and into secondary lymphoid organs (SLO) where the lymphocytes get activated. If they do not get activated in the SLO they continue circulating between blood and lymph. The CCR7 ligand CCL21 lets T cells enter secondary lymphoid organs as this chemokine can activate the integrin needed for the T cell to adhere to the SLO and enter via high endothelial venules (HEV) (Stein and Nombela-Arrieta 2005). Naïve B cells use either CCR7 or CXCR4 to adhere and enter the SLO (Okada, Ngo et al. 2002). Once in the

SLO, CCL19 and CCL21 keep the T cells in the T cell area whereas CXCL13 retains B cells, which constitutively express the CXCL13 receptor CXCR5, in the B cell follicles. After antigen contact, activated B cells are more responsive to the CCR7 ligands CCL21 and CCL19 which help the activated B cells to migrate towards the T cell area to receive stimulatory signals from T cells (Kunkel and Butcher 2003). CXCL12 and CXCL13 also help activated B cells to form functioning germinal centers (Stein and Nombela-Arrieta 2005). Activated B cells also increase their responsiveness to the CCR6 ligand CCL20, which helps them to localize to the region in the Peyer's patches where antigen is transported in (Kunkel and Butcher 2003). Once the activated B cells are in the germinal centre, they downregulate their responsiveness to chemokines and undergo class-switching. Some of these germinal centre B cells will develop into plasma cells, which lose their expression of CXCR5 and CCR6. This loss of responsiveness seen in plasma cells most likely facilitates their exit from the SLO. On the other hand, some class-switched B cells will develop into memory cells in which case they regain responsiveness to CCR7, CXCR5, CCR6 and CXCR4 ligands (Kunkel and Butcher 2003).

Inflammation. Chemokines are also involved in controlling the migration of leukocytes into inflamed tissue. Infiltration into tissue by effector leukocytes occurs in chronic diseases as well as immune responses against pathogens. The release of inflammatory molecules at the site of inflammation results in the up-regulation of adhesion molecules and display of chemokines at the luminal side of activated endothelial cells. All these molecules act in concert to arrest and bind leukocytes to the endothelium so they can

move through the endothelium and to the site of inflammation (Luster 1998). T_H1 effector cells show preferential expression of CCR5 and CXCR3 to migrate to the site of inflammation where the respective chemokines are produced (Moser and Loetscher 2001). CXCR3 is also expressed on plasma cells and a fraction of memory B cells but is absent from naïve B cells and as for T cells CXCR3 helps these cells to migrate to sites of inflammation (Kunkel and Butcher 2003; Manz, Moser et al. 2006). Additionally, expression of CCR6, which is also expressed by naïve B cells but is only functional after B cell receptor cross-linking, might help memory B cells to migrate to sites of inflammation where there is increased release of CCR6 ligand CCL20 (Kunkel and Butcher 2003).

Angiogenesis. Chemokines can influence angiogenesis positively or negatively. ELR^+ CXC chemokines induce angiogenesis whereas ELR^- CXC chemokines inhibit new vessel formation. However, there are exceptions such as CXCL12, which despite its lack of the ELR motif has an angiogenic function (Le, Zhou et al. 2004).

1.3 B CELLS IN RHEUMATIC AUTOIMMUNE DISEASE

B cells contribute to rheumatic autoimmune diseases such as RA and systemic lupus erythematosus (SLE) by turning into autoantibody-producing plasma cells. Autoantibodies are responsible for at least part of the pathology seen in RA and SLE, as both diseases show immune complex mediated tissue injury (Lipsky 2001; Takemura, Klimiuk et al. 2001). Edwards and Cambridge proposed a model for RA of autoantibody-secreting B cells that start a cycle of 'self-perpetuation' in which the autoantibody

(rheumatoid factor in RA) provides a positive feedback signal to the autoreactive B cell. IgG rheumatoid factor can fix complement (C3d) thus co-ligating complement receptor CR2 and the autoreactive B cell receptor. Rheumatoid-factor producing B cells can present foreign antigen to non-autoreactive T cells, which in turn would provide the help to the autoreactive B cell (Edwards and Cambridge 2006).

Still, a central role for $CD4^+$ T cells and macrophages in disease pathology has been suggested in RA as autoantibodies do not correlate with disease activity and RA does also occur in individuals negative for rheumatoid factor (Takemura, Klimiuk et al. 2001; Dorner 2006). However, as described in the following paragraphs, the role of B cells in rheumatic autoimmune diseases is more complex.

B cells and ectopic lymphoid neogenesis. B cells are part of the immune cell infiltrate in the inflamed synovium in RA. B cells can just form a diffuse infiltrate or they can organize themselves into aggregates with T cells, but lacking follicular dendritic cells and germinal centers (Weyand, Seyler et al. 2005). However, in 10 to 23% of RA cases they actually form fully developed germinal centers (GC), which are sites of B cell proliferation and maturation and usually only occur in secondary lymphoid organs after B cell activation. These GC also include T cells and follicular dendritic cells thus forming an organized structure where activated B cells can locally differentiate into antibody-secreting plasma cells (Magalhaes, Stiehl et al. 2002). The formation of such organized structures of lymphoid cells is called ectopic lymphoid neogenesis and the GC are referred to as ectopic GC. The finding of these ectopic GC shows that B cell activation including somatic hypermutation can take place in the nonlymphoid tissue of the

synovium in RA patients (Schroder, Greiner et al. 1996). Thus, it illustrates the involvement of B cells in the chronic inflammatory process of the disease and highlights the role of lymphoid neogenesis in the promotion of the autoimmune reaction.

Furthermore, B cells contribute to the development of ectopic lymphoid structures by producing lymphotoxin $\alpha_1\beta_2$ (Takemura, Braun et al. 2001). TNF-family members such as lymphotoxin $\alpha_1\beta_2$, are required for the development and formation of SLO and subsequently for lymphoid neogenesis (Takemura, Braun et al. 2001; Aloisi and Pujol-Borrell 2006). Additionally, lymphotoxin is needed for the differentiation of follicular dendritic cells in SLO (Lipsky 2001).

B cells as antigen-presenting cells (APC). A study by Takemura et *al.* showed that T cell activation in rheumatoid synovitis is actually B cell-dependent (Takemura, Klimiuk et al. 2001). The authors concluded that these synovial B cells most likely function as APC for the T cells as they might be superior in capturing antigen in the inflamed synovium compared to other APC. This could be the case especially if antigen concentrations are limited (Takemura, Klimiuk et al. 2001).

B cells have also been identified as APC in a murine model of RA, namely collagen-induced arthritis. Using B cell-deficient mice Taneja et *al.* could show that B cells efficiently present antigen to autoreactive T cells (Taneja, Krco et al. 2007).

Co-stimulatory signals by B cells. Activated B cells can also provide co-stimulatory signals to the T cell. Upon activation of the B cell, OX40L expression is up-regulated and OX40L binds to OX40 expressed on the activated CD4 T cell. This interaction is enough to initiate Th2 differentiation of the T cell (Flynn, Toellner et al. 1998).

Cytokine production by B cells. B cells are known to produce cytokines such as IL-6, TNF-α and IL-10. Furthermore, naïve B cells can be induced to produce immunoregulatory cytokines such as IL-2, IFN-γ, IL-12 and IL-4 upon stimulation with antigen and polarized effector T cells thus turning into 'effector' B cells (Harris, Haynes et al. 2000). These 'effector' B cells can regulate naïve T cell development into effector T cells (T_h1 or T_h2) while acting as an APC. Also, activated B cells secrete cytokines, which can influence the function of dendritic cells as APC (Lipsky 2001).

1.3.1 BAFF in B cell autoimmunity

The B cell activating factor BAFF (also referred to as BLyS, THANK, TALL-1, TNFSF13b and zTNF4) is important for B cell activation and survival. It belongs to the TNF superfamily and is expressed by monocytes, macrophages, dendritic cells, neutrophils, stromal cells and activated T cells (Ramanujam and Davidson 2004; Seyler, Park et al. 2005). BAFF is a type II transmembrane protein but it can be cleaved from the cell membrane and thus become soluble, either in a homotrimeric or heterotrimeric form (Mackay, Schneider et al. 2003). BAFF binds to three different receptors, BAFF-R (BAFF-receptor), BCMA (B cell maturation antigen) and TACI (transmembrane activator and calcium ligand interactor). BAFF-R is expressed by mature B cells and memory B cells whereas BCMA is expressed highest by plasma cells. TACI is expressed by mature B cells and activated T cells (Ramanujam and Davidson 2004; Seyler, Park et al. 2005). TACI and BCMA also bind the BAFF homologue APRIL (a proliferation-inducing agent). APRIL signalling results in proliferation, class switching and survival of

B cells but it is not essential for B cell development as APRIL knock-out mice do not have defects in their immune system (Baker 2004; Ramanujam and Davidson 2004). APRIL can also form heterotrimers with BAFF but the physiological significance of these trimers is not fully understood (Baker 2004).

BAFF stimulates B cell proliferation in *vitro*, but it acts as co-stimulatory signal only in combination with B cell receptor (BCR) cross-linking (Baker 2004). BAFF is important for B cell survival in the spleen during peripheral maturation from the T2 transitional stage onwards and for development of marginal zone B cells (Mackay, Schneider et al. 2003). Mice that do not express BAFF lack mature B cells and blocking BAFF in adult mice leads to the depletion of mature B cells thus showing their dependence on BAFF-mediated survival signals (Mackay, Schneider et al. 2003; Brink 2006). These survival signals are mediated via BAFF-R (Mackay, Schneider et al. 2003). TACI on the other hand functions as a negative regulator of B cells and BCMA plays a role in the long-term survival of plasma cells. Transgenic overexpression of BAFF in mice results in a SLE-like disorder (Gross, Johnston et al. 2000). Also, mice of the NZWxBF$_1$ and MRL-*lpr/lpr* mouse models of SLE show elevated levels of BAFF and the development of disease in the NZWxBF$_1$ mice could be inhibited by administration of a soluble BAFF antagonist (Gross, Johnston et al. 2000). BAFF antagonists have been shown to prevent arthritis in the DBA1 mouse model of collagen-induced arthritis (Ramanujam and Davidson 2004). These findings in animal models suggest that BAFF levels might play a role in human autoimmune diseases. Elevated serum levels of BAFF have since been shown among others in SLE patients and about 20% of RA patients show increased serum BAFF levels

(Cheema, Roschke et al. 2001). In an effort to elucidate the role of BAFF in sustaining B cell function in arthritis, Seyler et *al.* showed a possible role for BAFF and its homolog APRIL in the formation and maintenance of ectopic germinal centers in the inflamed synovium of rheumatoid arthritis patients (Seyler, Park et al. 2005).

1.3.2 B cell depletion therapy

As B cells play a number of roles in RA disease pathogenesis, B cell depletion therapy has become a new focus for RA therapy. This is especially important for patients unresponsive to anti-TNF treatment. The drug of choice for B cell depletion therapy is Rituximab, a chimeric anti-CD20 monoclonal antibody consisting of a human IgG constant region and the variable region of a murine anti-CD20 antibody (Chambers and Isenberg 2005). CD20 is expressed on B cells from the pre-B cell stage onwards but is absent from plasma cells (Cope and Feldmann 2004). Rituximab depletes B cells mainly by antibody-dependent cell-mediated cytotoxicity (ADCC) but also by fixing complement leading to B cell lysis and by promoting B cell apoptosis (Edwards and Cambridge 2006). Rituximab was originally used for the treatment of Non-Hodgkin's lymphoma. Early case reports of lymphoma patients receiving Rituximab treatment showed a remission of coexisting RA. This led to a small exploratory study of Rituximab use in RA patients that were unresponsive to at least five DMARDs (Shaw, Quan et al. 2003). With more successful trials completed, Rituximab received FDA approval for the treatment of RA in 2006. A small study including 20 patients showed the efficacy of Rituximab in a 'real life' setting as the enrolled patients had long-standing RA and had

failed anti-TNF therapy. Two infusions of Rituximab were enough to sustain improvement for 6 months and re-treatment was only necessary at 6 to 18 months (Jois, Masding et al. 2007). Generally Rituximab is well tolerated with the most common adverse event being infusion reactions (Chambers and Isenberg 2005). Rituximab therapy is now also under trial in other autimmune diseases such as SLE, immune thrombocytopaenia and multiple sclerosis (Edwards and Cambridge 2006).

B cells can also be targeted by neutralizing survival factors such as BAFF. As mentioned above, the inhibition of BAFF by administration of antagonists delayed disease onset in SLE-prone mice and inhibited collagen-induced arthritis in mice (Ramanujam and Davidson 2004; Cambridge, Stohl et al. 2006). Furthermore, elevated serum BAFF levels have been found in patients with SLE and RA (Cambridge, Stohl et al. 2006). Thus, BAFF inhibition has been studied in SLE and RA patients using a monoclonal antibody against BAFF called Belimumab (Edwards and Cambridge 2006; Isenberg 2006). Belimumab produced a decrease in autoantibody levels as well as moderate B cell depletion in RA patients; in SLE patients there was a 12% to 47% reduction in B cells (Edwards and Cambridge 2006; Isenberg 2006). Belimumab does not seem to be as clinically effective as Rituximab, but animal studies indicate that Belimumab might act synergistically with anti-CD20 agents (Edwards and Cambridge 2006; Isenberg 2006).

1.4 RATIONALE AND OBJECTIVES

The role of B cells in rheumatic autoimmune disease has been studied primarily in RA and its animal models, as described in section 1.3. In addition to producing autoantibodies B cells can function as APC, cytokine producers and providers of co-stimulation. With the successful use of B cell depletion therapy in RA, the interest has shifted to B cells as contributors to disease pathology. Although RA and PsA are distinct clinical conditions, each having unique characteristics, they share features such as polyarticular joint involvement and similar histology of the inflamed joint. Thus the evidence for the involvement of B cells in RA pathology serves as a possible indicator of mechanisms that may also occur in PsA.

Research in PsA immunology has so far been focused on T cells due to several reasons. Firstly, the majority of PsA patients are considered seronegative for autoantibodies like rheumatoid factor and anti-cyclic-citrullinated-peptide antibodies (Cassell and Kavanaugh 2005). Also, as described in section 1.1.3 b), psoriasis is considered to be a T-cell-mediated disease. Consistent with the association of PsA primarily with HLA class I genes, $CD8^+$ T cells are the most abundant lymphocytes found in the synovial fluid and in the epidermis from PsA patients.

There is evidence for B cell involvement in PsA. A study by Veale et *al.* showed that B cells and plasma cells were present in skin biopsies taken from PsA patients with psoriasis but not in samples taken from psoriasis patients with no arthritis and healthy controls (Veale, Barnes et al. 1994). The presence of B cells in the inflamed synovium was also shown by Gerhard et *al.* (Gerhard, Krenn et al. 2002). Their IgVH analysis of

B cells from the inflamed synovium demonstrated that the B cells are actually antigen-activated. The infiltrating B cells formed aggregates in a follicle-like pattern but follicular dendritic cells were absent thus indicating that there was no germinal center reaction present. Still, a few IgVH genes showed a clonal relationship and somatic hypermutation, which is indicative of a local germinal center reaction. The authors concluded that these clonally related IgVH genes might be the remainders of a previously present germinal center as germinal centers can be transient.

Canete et *al.* further studied the role of B cells in PsA and showed the presence of ectopic lymphoid neogenesis in PsA synovium (Canete, Santiago et al. 2007). Several studies have previously found lymphoid neogenesis and ectopic germinal centers in RA (see section 1.3). 60% of tested PsA synovial tissues had large lymphoid aggregates with T and B cell segregation. The authors also investigated the presence of other features typically present in SLO. They found vessels with high endothelial venule (HEV) morphology and showed that the SLO typical chemokines CXCL13 and CCL21 were expressed as well, however only in the tissues containing large aggregates. Unfortunately, the presence of follicular dendritic cells was not studied.

About one third of PsA patients do not respond to the treatment with TNF-α inhibitors (Cassell and Kavanaugh 2005) and thus there is a need for new therapeutic options. As there is little known about the role of B cells in PsA pathology, apart from the studies mentioned above, the aim of the present study was to provide supporting evidence of B cell involvement in PsA. This was done in two parts, the first focusing on the migration of peripheral blood B cells. Since chemokines are vital for lymphocyte migration, we

hypothesized that there may be a differential expression of chemokine receptors on peripheral blood B cells that would allow B cells from patients with inflammatory arthritis to be recruited to the inflamed tissues. To test this hypothesis the expression of seven chemokine receptors was determined on peripheral blood B cells from PsA and RA patients as well as healthy controls using flow cytometry and RT-PCR and compared between each group. The second part of the study focused on determining B cell surviving factor BAFF levels in plasma, which has not been previously reported for PsA patients. Serum BAFF levels are known to be elevated in patients with rheumatic autoimmune disorders such as RA and SLE and are thought to contribute to the survival of autoreactive B cells in these diseases (Cheema, Roschke et al. 2001; Lesley, Xu et al. 2004). If similarly elevated BAFF levels occur in PsA patients, this would add further weight to support the role for B cells in PsA disease pathology. This hypothesis was tested by analyzing the plasma BAFF levels by ELISA in PsA and RA patients and comparing these to BAFF levels in healthy controls.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Reagents used for agarose gel electrophoresis were purchased from Invitrogen (Burlington, Canada) and GE Healthcare (Baie d'Urfé, Canada). Cell culture reagents like fetal bovine serum (FBS) and antibiotics were obtained from Invitrogen (Burlington, Canada) and Sigma Aldrich (Oakville, Canada).

2.1.2 Molecular biology reagents

All reagents for RT-PCR including RNA isolation, cDNA synthesis and PCR were purchased from Invitrogen (Burlington, Canada). Chloroform and other chemicals were obtained from Sigma Aldrich (Oakville, Canada). PCR primers were either purchased from R&D Systems (Minneapolis, USA) for the chemokine receptor assays or Integrated DNA Technologies (Coralville, USA) for the ß-actin, CXCR3 and CD3 assays. Dr. Thomas Michalak kindly provided the plasmid used as positive control for the ß-actin assay. The plasmids for the CXCR3 and CD3 assays were produced and provided by Shashi Gujar (Memorial University of Newfoundland).

2.1.3 Antibodies for flow cytometry

All antibodies against human chemokine receptors were purchased from R&D Systems (Minneapolis, USA). The antibody against CD19 (clone HIB19) was obtained from Biolegend (San Diego, USA).

2.1.4 Reagents for ELISA

For the sandwich ELISA a Quantikine human BAFF kit (Catalogue # DBLYS0) was purchased from R&D Systems (Minneapolis, USA). It contained all necessary reagents for the ELISA including pre-coated plates, human BAFF standard and horseradish peroxidase conjugate.

2.2 METHODS

2.2.1 Sample collection

Participants for the study were recruited at the Rheumatology clinic at Eastern Health in St. John's, Canada, after the study was approved by the Human Investigations Committee. All volunteers gave written consent to participate in the study. Patients with rheumatoid arthritis (RA) fulfilled the American College of Rheumatology criteria for RA.(Arnett, Edworthy et al. 1988) Psoriatic arthritis (PsA) patients fulfilled the Moll and Wright criteria as patients were recruited before August 2006 when the new CASPAR criteria were published (Moll and Wright 1973; Taylor, Gladman et al. 2006). The controls were ethnically matched, unrelated to the participating patients and had no autoimmune disease at the time of recruitment.

2.2.2 Isolation of peripheral blood mononuclear cells

For each sample, 40 mL of venous blood were collected in EDTA vacutainer tubes and processed within 2-3 hours of collection using Ficoll-Paque Plus (GE Healthcare, Baie d'Urfé, Canada). The following protocol was modified from the manufacturer's instructions. First, the blood was centrifuged at 400 g for 10-15 minutes at room temperature (23° C) to separate the fractions of the blood. Then the plasma layer was removed and stored at -80° C. The remaining blood sample was diluted with an equal amount of sterile PBS and slowly layered on top of Ficoll-Paque Plus (GE Healthcare, Baie d'Urfé, Canada) using Ficoll at ¾ the volume of the diluted sample. The sample was then centrifuged for 30-40 minutes at 400 g. The plasma phase was removed and also stored at -80° C. The buffy coat containing the peripheral blood mononuclear cells (PBMC) was transferred to a new tube and washed by adding three times the PBMC volume of sterile PBS. The Ficoll layer and the layer containing red blood cells and granulocytes were discarded. The diluted PBMC were centrifuged at 100 g for 10 minutes to pellet the cells. The cells were washed by removing the supernatant and resuspending the cells in 10 mL PBS containing 1% FBS. The cells were then centrifuged for 5 minutes at 100 g. Depending on the pellet size, the cells were resuspended in 10-12 mL PBS containing 1% FBS and counted using a hemacytometer (improved Neubauer cell). For the count the cells were diluted 1:2 with Trypan blue to exclude dead cells. Viability ranged between 84 – 99%. The cells were pelleted by centrifugation for 5 minutes at 100 g and resuspended in cooled freezing medium (RPMI 1640 containing 10% FBS and 10% DMSO). The PBMC were aliquoted and slowly frozen over night at

-80° C using a freezing container to ensure a -1° C/minute cooling rate. The sample aliquots were then transferred into a liquid nitrogen tank for long-term storage.

2.2.3 RT-PCR

a) B cell isolation

$CD19^+$ cells (B cells) were isolated from the whole PBMC population using the magnetic cell separation system EasySep (Stemcell Technologies, Vancouver, Canada). The protocol provided by the manufacturer was followed for the EasySep separation. Depending on the total number of isolated PBMC, $20 \times 10^6 - 50 \times 10^6$ total cells per sample were used for the magnetic cell separation. A modified version of the thawing protocol published by Hansen et *al.* was used (Hansen, Reiter et al. 2005). First, the frozen PBMC were thawed in a 37° C water bath and an equal amount of chilled thawing medium (RPMI 1640 containing 20% FBS, 1% glutamine and 2% penicillin and streptomycin) was added to the thawed sample. Then the cells were centrifuged for 5 minutes at 100 g and resuspended in 2-3 mL of warm thawing medium and incubated for 15 minutes at room temperature (23° C). Cell viability was again determined using Trypan blue and the cells were counted in an improved Neubauer hemacytometer. Cell viability ranged between 80 – 97%. The cells were pelleted for 5 minutes at 100 g and resuspended in 100 µL EasySep medium (PBS containing 2% FBS and 1mM EDTA) before being transferred to a 12 x 75 mm polystyrene tube. EasySep Positive Selection Cocktail containing monoclonal antibodies against CD19 was added at 100 µL/mL cells

(10 µL for 100 µL cells) and the mixture was incubated for 15 minutes at room temperature (23° C). Then EasySep Magnetic Nanoparticles were added at 50 µL/mL cells (5 µL for 100 µL cells) and incubated at room temperature for 10 minutes. Then the appropriate amount of EasySep medium was added to bring the cell suspension to a total volume of 2.5 mL. The suspension was mixed by pipetting and put in the magnet for 5 minutes. Next, the supernatant fraction was poured off with the tube still in the magnet to keep the magnetically labeled cells in the tube. The positively selected B cells in the tube were then washed 3 times by adding 2.5 mL EasySep medium to the tube and left in the magnet for 5 minutes each time. The supernatant fractions (containing T cells, macrophages and NK cells) from each step were collected and pooled. The $CD19^-$ cells were counted using an improved Neubauer hematocytometer and centrifuged for 10 minutes at 100 g before being resuspended in freezing medium (RPMI 1640 containing 10% FBS and 10% DMSO). The cells were aliquoted and frozen as described above. The positively selected B cells were counted and then used for subsequent RNA isolation.

To assess the purity of the positively selected fraction, CD19-PE (Biolegend, San Diego, USA) antibody was added at a concentration of 0.15 µg/mL immediately after adding the EasySep Positive Selection Cocktail. The following separation of the $CD19^+$ cells was performed as described above. Then the positively selected cells were analyzed using a flow cytometer (see 2.2.4 b). 94% of the positively selected cells were indeed $CD19^+$ B cells (10,000 total events). In another purity assessment the $CD19^+$ fraction was stained

using CD20-FITC (Biolegend, San Diego, USA) as described in section 2.2.4 a. Here 97% of the enriched fraction was CD20 positive.

b) RNA isolation

The protocol for RNA isolation using TRIzol (by Invitrogen, Burlington, Canada) was adapted from Pham et *al.* (Pham, MacParland et al. 2004) The separated B cells were lysed in 1000 µL TRIzol and incubated for 30-60 minutes at room temperature (23° C). For the extraction of RNA, 200 µL chloroform were added and the sample was shaken for 15 seconds followed by a 2-3 minute incubation period at room temperature (23° C). Then the sample was centrifuged at 12,000 g for 15 minutes at 4° C. The upper aqueous phase containing the RNA was transferred into a new tube containing 500 µL chilled isopropanol. RNA was precipitated overnight at -20° C using 2 µL of glycoblue solution (15 µg/ µL) as co-precipitation material. Next, the RNA was pelleted by centrifugation at 12,000 g for 10 minutes at 4° C. The RNA pellet was then washed using chilled 1000 µL 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4° C. The supernatant was removed and the RNA pellet was air-dried for about 10 minutes. After resuspension in 10 µL RNase-free water the RNA was left on ice for 30 minutes. Then the RNA pellet was heated for 10 minutes at 55° C to completely dissolve the pellet. The RNA concentration was determined spectrophotometrically at a wavelength of 260 nm (Warburg-Christian method). The RNA was stored at -80° C before being used for cDNA synthesis.

c) cDNA synthesis

For the cDNA synthesis, 7 µL of total RNA (varying between 0.40 µg – 1.69 µg) were used for a 20 µL reaction volume; a tube containing all the reagents except an RNA template served as a negative control. All reagents were purchased from Invitrogen (Burlington, Canada) and the manufacturer's protocol was followed. 125 ng random primers and 1 µL of a 10 mM dNTP mix were added and the reaction heated at 65° C for 5 minutes. After being shortly chilled on ice, 4 µL of 5x first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µL of 0.1 M DTT and 40 units of RNaseOUT Recombinant Ribonuclease Inhibitor were added to the mixture. The reaction was then incubated at 37° C for 2 minutes. 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) were used for the cDNA synthesis and the reaction was incubated for 10 minutes at room temperature (23° C). Next, the mixture was heated for 40 minutes at 37° C and then inactivated by heating at 70° C for 15 minutes. The cDNA was stored at -20° C before being used in a PCR assay.

d) PCR for chemokine receptors

2 µL cDNA were used for each sample for a first amplification of the house-keeping gene β-actin. After the first β-actin PCR, the cDNA volumes for each sample were adjusted depending on the band size from 1 – 2.5 µL cDNA to compensate for the varying amounts of template RNA. The adjusted cDNA volumes were then used for a second β-actin PCR and subsequent assays for the chemokine receptors and CD3. The PCR reaction was carried out following the protocol supplied by R&D Systems.

For a 50 µL PCR reaction volume, 5 µL of PCR buffer (containing 200 mM Tris-HCl at pH 8.4 and 500 mM KCl), 1 µL 10 mM dNTP mix, 1.5 µL 50 mM $MgCl_2$, 1 µL of each β-actin primer at 10 µM and 2.5 units of *Taq* DNA polymerase were added to the cDNA. A tube containing water instead of cDNA served as negative control for the reaction, a β-actin plasmid (provided by Dr. Thomas Michalak) as a positive control. The β-actin primers were 5'-CAA CCG TGA GAA GAT GAC C-3' forward and 5`-ATC TCC TGC TCG AAG TCC –3' reverse (kindly provided by Dr. Thomas Michalak). Next, the reaction was heated in a thermal cycler first for 5 minutes at 94° C followed by 35 cycles of 94° C denaturing for 45 seconds, 55° C annealing for 45 seconds and 72° C extension for 45 seconds and lastly 10 minutes at 72° C.

The PCR products were visualized using agarose gel electrophoresis. The β-actin product had a size of 339 bp and was run on a 1.5% agarose gel. Ethidium bromide solution was added to a final concentration of 0.5 µg/mL to the agarose mixture before pouring the gel. 10 µL of PCR product were mixed with 2.5 µL BlueJuice loading buffer (Invitrogen, Burlington, Canada) and loaded on the gel. The gel was run in a mini-sub cell GT electrophoresis cell (Biorad) for about 1 hour at 80 V. The bands were then visualized under UV light and analyzed using the Kodak Molecular Image software. The band net intensity was determined and the band intensities of the chemokine receptors and CD3 were normalized against the corresponding band intensities of β-actin.

The same procedure was followed for the PCR reactions of all the chemokine receptors. The primers for the receptors CXCR4 (cat. # RDP-261-025), CXCR5 (cat. # RDP-243-025), CCR1 (cat. # RDP-206-025), CCR2 (cat. # RDP-207-025), CCR5 (cat. # RDP-213-

025) and CCR6 (cat. # RDP-237-025) were obtained from R&D Systems (Minneapolis, USA), which also included a positive control for each primer supplied. The primers were used at a concentration of 7.5 μM each in the PCR reaction. The primers for CXCR3 were 5'- CCA CCC ACT GCC AAT ACA AC –3' forward and 5'- CGG AAC TTG ACC CCT ACA AA –3' reverse as previously described by Huber *et al.* (Huber, Reinhardt et al. 2002) and purchased from Integrated DNA Technologies (IDT, Coralville, USA). The CXCR3 primers were used in the PCR reaction at a concentration of 10 μM each. As described by Huber *et al.*, the annealing temperature for these primers was increased to 60° C and only 30 cycles were run. The primers for the CD3 assay were 5'- TGA GGG CAA GAG TGT GTG AG –3' forward and 5' –GAG GCA GTG TTC TCC AGA GG –3' reverse and also used at 10 μM each primer. The primers were designed with the Primer3 program (http://frodo.wi.mit.edu/) using a human CD3ε mRNA sequence (accession number NM_000733). The expected CD3 product was used in a BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat) to test for potential genomic DNA products. No genomic DNA products with the same size as the cDNA product should be observed. The plasmids used as positive controls for CXCR3 and CD3 were produced and provided by Sashi Gujar. Table 2.1 lists the cDNA product sizes for the PCR assays analyzed.

Table 2.1: cDNA product sizes for analyzed PCR assays

PCR assay	cDNA product size	Genomic DNA product size	Positive control product size
CCR1	201 bp	-	320 bp
CCR2	406 bp	-	320 bp
CCR5	459 bp	2363 bp	320 bp
CCR6	482 bp	579 bp	350 bp
CXCR3	602 bp	602 bp	330 bp
CXCR4	724 bp	-	330 bp
CXCR5	253 bp	-	350 bp
CXCR3	379 bp	-	379 bp
CD3	305 bp	-	305 bp
β-actin	339 bp	-	339 bp

2.2.4 Flow cytometry

a) Staining for flow cytometry

All antibodies against the chemokine receptors (CXCR3, CXCR4, CXCR5, CCR1, CCR2, CCR5 and CCR6) as well as the matching mouse IgG2b and IgG1 isotype controls were PE-conjugated and purchased from R&D Systems (Minneapolis, USA). The antibody against CD19 and the matching mouse IgG1 isotype control were allophycocyanin (APC)-conjugated and obtained from Biolegend (San Diego, USA). The procedure was adapted from R&D Systems. For the flow cytometry staining 5 x 10^6 – 10 x 10^6 PBMC were thawed as described under B cell isolation. After counting the cells and the last centrifugation step the PBMC were resuspended in the appropriate amount of PBS containing 1% FBS to get a final concentration of 5x10^6 cells/mL. For each sample, 400,000 cells was double-stained for each chemokine receptor and CD19. The antibodies were titrated in a previous experiment to determine the optimum antibody concentrations, which are summarized in Table 2.2. After adding the appropriate amount of antibody to each tube, 80 µL of PBMC were added to the tube. In separate tubes the appropriate isotype controls were set up as negative controls (negative control 1: mouse IgG1-APC and IgG1-PE; negative control 2: mouse IgG1-APC and IgG2b-PE) and again 80 µL of PBMC were added to the tubes. Then the tubes were incubated for 30 minutes at 4° C in the dark. After the incubation time the stained cells were washed with 1 mL of chilled PBS containing 1% FBS and centrifuged for 5 minutes at 100 g. This washing step was repeated twice for a total 3 washes.

Table 2.2: Antibodies used for flow cytometry staining and their optimum concentrations

Antibody	Clone	Conjugate	Isotype	Amount used for staining
CD19	HIB19	APC	Mouse IgG1	0.25 µg
CXCR3	49801	PE	Mouse IgG1	0.5 µg
CXCR4	44717	PE	Mouse IgG2b	0.5 µg
CXCR5	51505.111	PE	Mouse IgG2b	0.33 µg
CCR1	53504	PE	Mouse IgG2b	0.66 µg
CCR2	48607	PE	Mouse IgG2b	1 µg
CCR5	CTC5	PE	Mouse IgG1	0.33 µg
CCR6	53103	PE	Mouse IgG2b	0.66 µg

Following the last washing step the cells were resuspended in 150 µL chilled PBS containing 1% FBS. The cells were then fixed by slowly adding 150 µL of chilled 2% paraformaldehyde. The cells were analyzed within 2-7 days after fixation.

b) Flow cytometry analysis

The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada) and CellQuest Pro software. First, a forward scatter vs. side scatter plot was created to identify the lymphocyte population according to size and granularity. This lymphocyte population was then gated and 50,000 events were collected in this gate. Next the chemokine receptor expression was determined for the $CD19^{+}$ population of the gated lymphocytes. Only $CD19^{high}$ cells were included in the analysis (see Fig. 2.1 for example on gated population). For this expression analysis, the histogram of the isotype control was subtracted from the histogram of the chemokine receptor. The analysis of the subtracted histogram gave the percentage of chemokine receptor-positive cells as well as the mean fluorescence intensity for the expression.

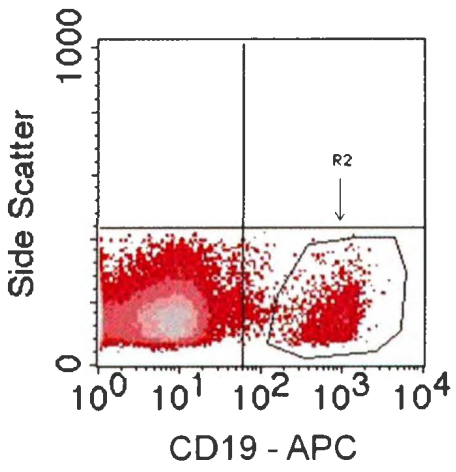


Figure 2.1: Gating for $CD19^+$ cells.

This is a representative density plot to show the gating strategy for B cells. After gating for the lymphocyte population, a second gate (R2) was used to gate for $CD19^{high}$ cells. Only those cells were used for chemokine receptor expression analysis.

2.2.5 ELISA

The Quantikine human BAFF kit from R&D Systems (Catalogue # DBLYS0) contained all the necessary reagents for the ELISA including pre-coated plates, human BAFF standard and horseradish peroxidase conjugate. First 100 µL/well of the supplied assay diluent were added to the plate followed by 50 µL of undiluted plasma or standard. The standard curve covered a range from 0.0625 ng/mL to 4 ng/mL. The plate was then incubated for 2 hours at room temperature and then washed 4 times using the provided washing buffer. Then 200 µL/well of HRP conjugate were added and the plate was again incubated for 2 hours at room temperature. After another 4 washes, 200 µL/well of provided substrate solution were pipetted into each well and the plate was incubated for 30 minutes at room temperature in the dark. The reaction was stopped using 50 µL/well of 2 N sulfuric acid. As recommended in the manufacturer's instructions, the plate was read at 450 nm with 550 nm as reference wavelength to correct for optical imperfection on the plate. By linear plotting of the optical density (mean of duplicates) against the corresponding standard concentration, a standard curve was generated. A linear trendline was added which was used to calculate the concentration in the plasma samples.

2.2.6 Statistical analysis

All statistical analysis was performed using Graphpad Prism 4.0. To compare the expression levels between the three groups (healthy control, RA, PsA) for both the chemokine receptor expression and the BAFF ELISA a nonparametric ANOVA (Kruskal-Wallis test) was used to determine significant differences in expression. A

Dunn's post test, which calculates a p-value for each pair of columns, was performed to determine which groups showed significant differences compared to the other groups. A p-value below 0.05 was considered significant. To test data for correlation with clinical parameters (BAFF ELISA data) or CD3 expression (chemokine receptor expression data) the Spearman test was performed.

CHAPTER 3

COMPARISON OF CHEMOKINE RECEPTOR EXPRESSION BY PERIPHERAL BLOOD B CELLS FROM PATIENTS WITH PSORIATIC AND RHEUMATOID ARTHRITIS

3.1 Introduction and rationale

Lymphocyte trafficking throughout the body is guided by chemotactic factors such as chemokines, which exert their function via their respective chemokine receptors. Thus lymphocytes can home to different sites of the body according to the expression profile of adhesion molecules and chemokine receptors of various tissues (Burman, Haworth et al. 2005). This 'area code model' enables lymphocytes to migrate to sites of inflammation where the corresponding chemokines are expressed to attract lymphocytes to the site. As described in Chapter 1, B cells are part of the cellular infiltrate into the PsA synovium and the development of ectopic lymphoid structures in PsA has been shown. The aim of the work described here was to show a possible recruitment of peripheral blood B cells to sites of inflammation in PsA patients. We hypothesized that differential expression of chemokine receptors on peripheral blood B cells would allow these B cells to migrate to the inflamed joints. The following paragraph describes the chemokine receptors that were selected for expression analysis. Some of these receptors are not constitutively expressed by B cells but have been shown to play a role in arthritis and PsA and were therefore chosen for the study. Other receptors are constitutively expressed by B cells and were selected since they are important for B cell migration. Other constitutively expressed chemokine receptors such as CCR7 were not analyzed as only a limited amount of receptors could be analyzed for this study.

The inflammatory chemokine CCL5 is found in PsA synovium and is produced by infiltrating T cells (Konig, Krenn et al. 2000). CCR5, one of the receptors for CCL5, is expressed by infiltrating mononuclear cells in the synovial fluid in PsA and RA patients

(Mack, Bruhl et al. 1999), which possibly enables these cells to migrate to the inflamed joint where CCL5 is produced. Another receptor activated by CCL5 is CCR1. The role of CCR1 in arthritis has been shown using the collagen-induced arthritis mouse model where a CCR1 antagonist was effective in ameliorating disease severity and incidence (Plater-Zyberk, Hoogewerf et al. 1997). Also, a human CCR1 antagonist is in a phase II clinical trial for use as a treatment of rheumatoid arthritis (Charo and Ransohoff 2006). Another inflammatory chemokine, CXCL9, has also been detected by immunohistochemistry in the synovium of PsA patients and seems to work together with CCL5 to recruit T cells to the inflamed synovium (Konig, Krenn et al. 2000). The CXCL9 receptor CXCR3 has been shown to be expressed by effector T cells as well as plasma cells and a fraction of memory B cells, which helps these cells to migrate to sites of inflammation (Moser and Loetscher 2001; Kunkel and Butcher 2003; Manz, Moser et al. 2006). CCL2 is another inflammatory chemokine that has been found in increased amounts in the synovial fluid of PsA patients compared to patients with non-inflammatory arthritis; thus, the CCL2 receptor CCR2 was investigated in this study (Ross, D'Cruz et al. 2000). CCR2 is also expressed by mononuclear cells in the synovial fluid of RA patients (Mack, Bruhl et al. 1999), thus, like other chemokine receptors, it might be important for these cells to travel to the inflamed joint.

The chemokine receptors CXCR4, CXCR5 and CCR6 are receptors for homeostatic chemokines that are constitutively expressed by B cells. Activated B cells increase their responsiveness to the CCR6 ligand CCL20, which might help memory B cells to migrate to sites of inflammation that release increased amounts of CCL20 (Kunkel and Butcher

2003). Similarly, it has been shown in psoriasis that CCL20 is expressed by keratinocytes in lesional psoriatic skin but not in nonlesional and normal skin. Since memory T cells that home to the skin express high levels of CCR6, CCL20 expression might help recruit memory T cells to the psoriatic lesion (Homey, Dieu-Nosjean et al. 2000). As described in chapter 2, CXCR4 and CXCR5 are important for B cell migration into and within secondary lymphoid organs. CXCR4 and CXCR5 are also required for GC organization (Stein and Nombela-Arrieta 2005). Additionally, T cells found in RA synovium express high levels of CXCR4, which could explain their retention in the synovium as synovial endothelial cells produce CXCL12 (Buckley, Amft et al. 2000).

To test the hypothesis of a differential chemokine receptor expression on peripheral blood B cells from PsA patients, the expression of CXCR3, CXCR4, CXCR5, CCR1, CCR2, CCR5 and CCR6 was analyzed using RT-PCR and flow cytometry and compared to the expression on B cells from healthy controls and RA patients who served as disease controls.

3.2 Results

3.2.1 Patients

The RA patients recruited for the study fulfilled the American College of Rheumatology criteria for RA (Arnett, Edworthy et al. 1988) and the PsA patients fulfilled the Moll and Wright criteria (Moll and Wright 1973). Volunteers who were ethnically matched and had no known autoimmune disease when they entered the study were used as controls. The demographics of the study participants are summarized in Table 3.1.

Table 3.1: Demographics and treatment of patients and volunteers enrolled for study.

	PsA patients	**RA patients**	**Healthy controls**
Total number	37	24	35
Age			
Mean (range)	49 (21-72)	59 (38-79)	33 (20-54)
Sex			
Male	17	4	13
Female	20	20	22
Rheumatoid factor (IgM)	n.d.		n.d.
Positive		16	
Negative		8	
Active joint count			
Mild (5 joints and less)	28	12	n.a.
Moderate (6-9 joints)	5	6	
Severe (>10 joints)	4	6	
Treatment			
None	1		
NSAIDS only	3	2	n.a.
DMARDs	10[a]	13[c]	
Anti-TNF agents	9[b]	4[d]	
Prednisone	12	13	

[a]one patient additionally on anti-TNF agent, four patients additionally on prednisone
[b]two patients additionally on prednisone (but not MTX), two patients additionally on MTX (but not prednisone)
[c]two patients additionally on anti-TNF agent and prednisone, six patients additionally on prednisone
[d]two patients additionally on MTX and prednisone, one patient additionally on prednisone
n.d. not determined; n.a. not applicable

3.2.2 Summary of results

The RT-PCR results are summarized in Table 3.2 and the flow cytometry results are summarized in Table 3.3.

As described in Materials and Methods, a nonparametric ANOVA (Kruskal-Wallis test) was used to determine differences in chemokine receptor expression between the three investigated groups. This creates one p-value for comparing three groups and the subsequent post-test (Dunn's post-test) determines between which groups the significant difference lies. Thus, if there is no statistical significant difference, only one p-value is reported as all the p-values of the post-test are above 0.05.

Table 3.2: Summary of RT-PCR results.

Shown is the mean ratio including standard deviation (ratio is the receptor net intensity normalized against β-actin band net intensity).

	RT-PCR expression by B cells			
	HC	RA	PsA	p-value*
CXCR3	0.0568 ±0.032 SD (n = 5)	0.0372 ±0.024 SD (n = 5)	0.0184 ±0.005 SD (n = 5)	0.0598
CXCR4	1.2450 ±0.174 SD (n = 5)	1.140 ±0.120 SD (n = 3)	1.260 ±0.303 SD (n = 4)	0.661
CXCR5	0.5740 ±0.147 SD (n = 5)	0.5750 ±0.101 SD (n = 4)	0.3925 ±0.147 SD (n = 4)	0.2346
CCR1	0.0417 ±0.034 SD (n = 9)	0.0640 ±0.021 SD (n = 9)	0.0377 ±0.025 SD (n = 9)	0.0679
CCR2	0.1059 ±0.121 SD (n = 9)	0.0694 ±0.048 SD (n = 9)	0.0392 ±0.032 SD (n = 9)	0.3330
CCR5	0.0297 ±0.031 SD (n = 9)	0.0359 ±0.017 SD (n = 9)	0.0143 ±0.012 SD (n = 9)	0.0656
CCR6	0.2836 ±0.229 SD (n = 9)	0.2194 ±0.198 SD (n = 10)	0.1314 ±0.126 SD (n = 12)	0.2066

*p-values refer to comparison of expression levels from all three groups

Table 3.3: Summary of flow cytometry results.

Shown is the mean of percent positive B cells as well as mean fluorescence intensity.

	Percent positive B cells			
	HC (n = 10)	RA (n = 15)	PsA (n = 13)	p-value
CXCR3	77.47 ±5.84 SD	70.34 ±11.10 SD	70.83 ±12.62	0.2193
CXCR4	90.60 ±2.21 SD	90.82 ±4.69 SD	91.04 ±5.31 SD	0.5895
CXCR5	94.94 ±2.43 SD	93.89 ±2.58 SD	92.03 ±5.50 SD	0.3059
CCR1	3.84 ±1.03 SD	4.84 ±3.61 SD	5.50 ±2.62 SD	0.3833
CCR2	9.41 ±7.68 SD	7.69 ±5.37 SD	10.49 ±5.01 SD	0.1643
CCR5	3.65 ±1.59 SD	4.75 ±3.01 SD	4.29 ±2.67 SD	0.7864
CCR6	92.34 ±3.06 SD	89.52 ±5.14 SD	89.69 ±8.62 SD	0.2839
	Mean fluorescence intensity			
	HC	RA	PsA	p-value
CXCR3	143.1 ±28.64 SD	138.9 ±25.84 SD	179.6 ±69.13	0.0686
CXCR4	278.8 ±30.80 SD	284.5 ±61.17 SD	343.4 ±83.29 SD	0.1069
CXCR5	511.5 ±78.63 SD	479.5 ±71.20 SD	476.3 ±90.58 SD	0.3254
CCR1	n.a.	n.a.	n.a.	n.a.
CCR2	n.a.	n.a.	n.a.	n.a.
CCR5	n.a.	n.a.	n.a.	n.a.
CCR6	242.6 ±103.3 SD	213.2 ±82.17	252.8 ±102.9 SD	0.4824

n.a. = not applicable; p-values refer to comparison of all three groups

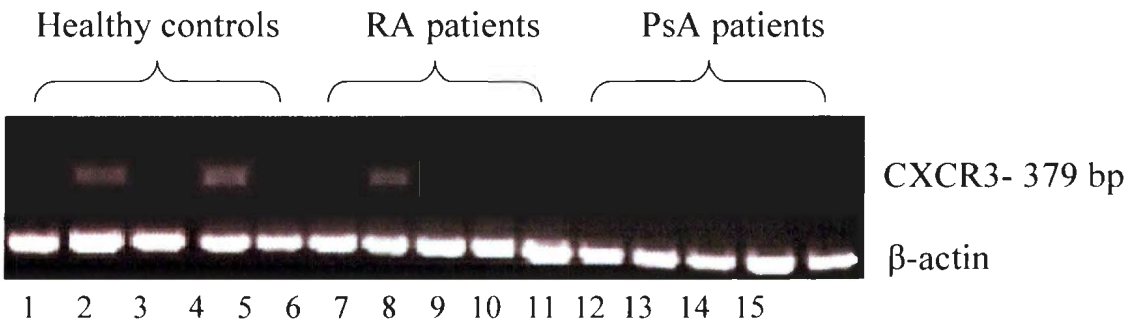
3.2.3 CXCR3 expression

a) RT-PCR

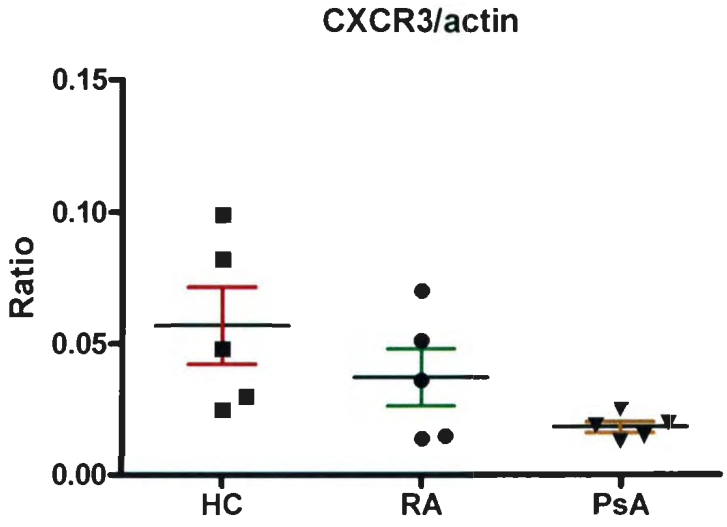
The expression of CXCR3 by peripheral blood B cells was first determined at the RNA level using RT-PCR. $CD19^+$ B cells were first isolated from PBMC using magnetic cell separation as described in Chapter 2 (Materials and methods) and then subsequently used for total RNA isolation, cDNA synthesis and PCR. A plasmid containing the human CXCR3 sequence served as positive control for the PCR. Five samples were analyzed for each of the investigated groups (PsA patients, RA patients and healthy controls). The PCR products were visualized using agarose gel electrophoresis and the net intensities of the CXCR3 product bands were normalized against the net intensities of the corresponding β-actin product bands (CXCR3/β-actin ratio).

As seen in Figure 3.1, isolated B cells from all groups expressed CXCR3 mRNA in low amounts. The PsA group showed the lowest expression with a mean ratio of 0.018 (± 0.002 standard error of mean) compared to healthy controls (0.057 ±0.015 SEM) and RA patients (0.037 ±0.010 SEM). Even though there was a trend visible, there was no significant difference between the three groups (p-value of 0.0598). There was also a large variation in CXCR3 mRNA expression within the RA group and healthy control group.

A


Healthy controls
RA patients
PsA patients
CXCR3- 379 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B

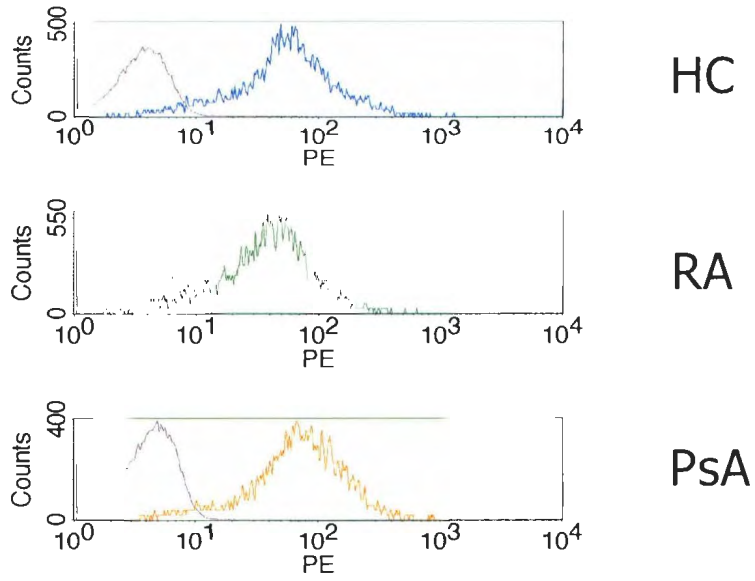

CXCR3/actin
Ratio
0.15
0.10
0.05
0.00
HC
RA
PsA

b) Flow cytometry

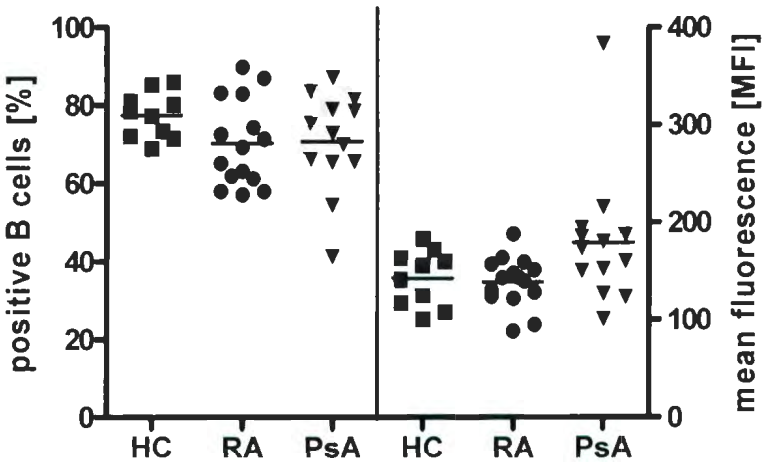
To see if a similar trend could be observed at the protein level, the expression of CXCR3 protein was also analyzed using flow cytometry. PBMC from 13 PsA patients, 15 RA patients and ten healthy controls were stained for CD19 and CXCR3. Then the CXCR3 expression of the gated $CD19^+$ population was determined by subtracting the isotype control histogram from the CXCR3 histogram. A density plot showing the gated population can be found in Chapter 2 (p. 54). The results of the chemokine receptor expression analysis are shown in Fig. 3.2. The majority of B cells did express CXCR3 and there was no significant difference ($p = 0.2193$) in the number of $CXCR3^+$ B cells between the three groups although RA (mean 70.34% ±2.87% SEM) and PsA patients (mean 70.83 ±3.50 SEM) showed a trend for a decrease in $CXCR3^+$ B cells compared to healthy controls (mean 77.47% ±1.85 SEM).

As determined by mean fluorescence intensity, despite most B cells expressing CXCR3, the B cells expressed it at lower levels compared to expression levels of other chemokine receptors such as CXCR4 and CXCR5. There was no significant difference in mean fluorescence intensity between the three groups ($p= 0.0686$) but the PsA group was the highest expressing group with a mean of 179.6 ±19.17 SEM compared to a mean of 143.1 ±9.06 SEM for healthy controls and a mean of 138.9 ±6.67 SEM for RA patients. In viewing Figure 3.2 B there clearly appears to be an outlier. Taking the outlier out of the analysis did not change PsA as the highest CXCR3-expressing group although the mean decreased slightly (from 179.6 to 162.6). Exclusion of the outlier did not result in a significant expression difference ($p = 0.1252$).

A


Counts
500
0
10^0 10^1 10^2 10^3 10^4
PE
HC
Counts
550
0
10^0 10^1 10^2 10^3 10^4
PE
RA
Counts
400
0
10^0 10^1 10^2 10^3 10^4
PE
PsA

B

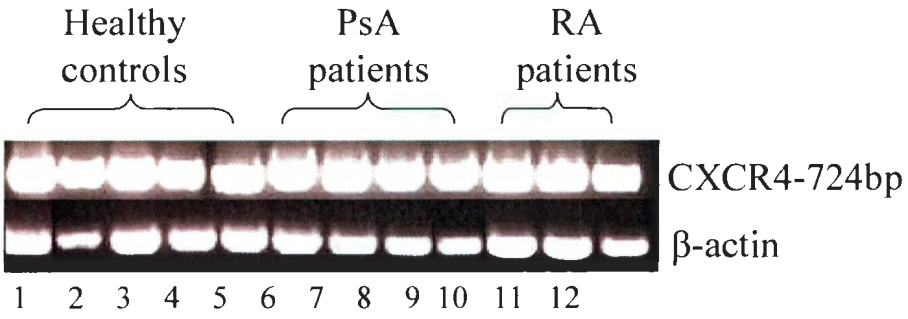

positive B cells [%]
100
80
60
40
20
0
HC
RA
PsA
HC
RA
PsA
400
300
200
100
0
mean fluorescence [MFI]

3.2.4 CXCR4 expression

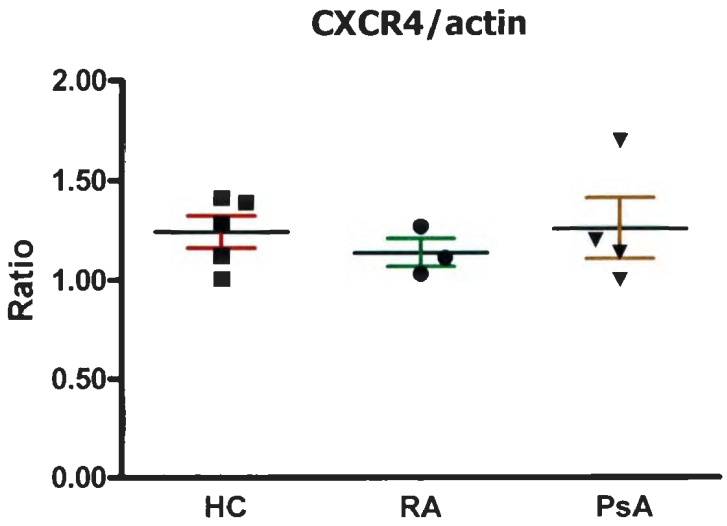
a) RT-PCR

The expression of CXCR4 was first determined using RT-PCR. Five samples from healthy controls, three samples from RA patients and four samples from PsA patients were used for analysis. The net intensities of the CXCR4 product bands were normalized against the net intensities of the corresponding β-actin product bands. There was no statistically significant difference in mRNA expression between the investigated groups ($p = 0.661$) and all groups expressed CXCR4 mRNA in high amounts as shown in Fig. 3.3. CXCR4 was the only receptor showing a stronger expression than the house-keeping gene β-actin with a ratio >1 for all groups.

A


Healthy controls
PsA patients
RA patients
CXCR4-724bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12

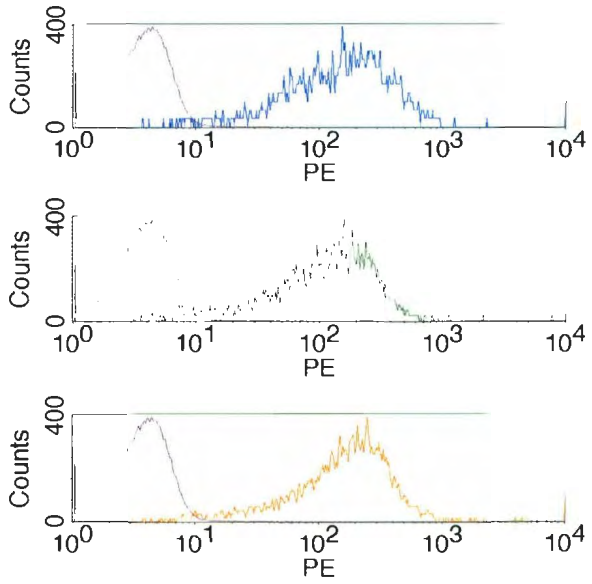
B


CXCR4/actin
Ratio
2.00
1.50
1.00
0.50
0.00
HC
RA
PsA

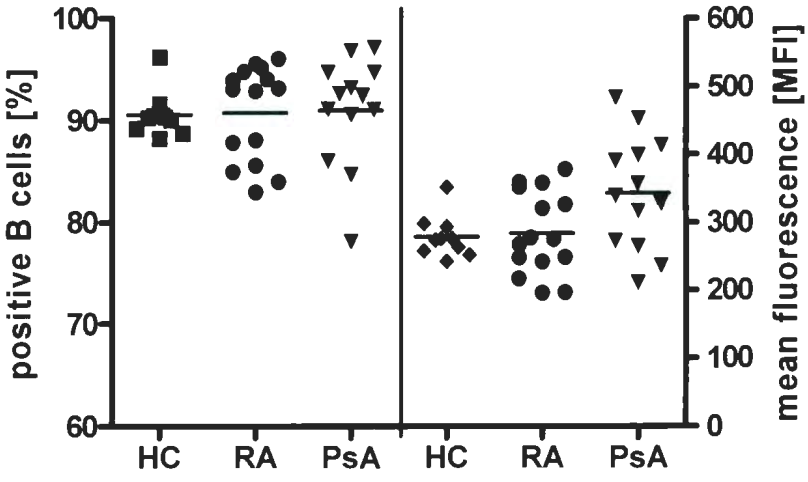
b) Flow cytometry

The expression of CXCR4 protein was also analyzed using flow cytometry. The results are shown in Fig. 3.4. Samples from ten healthy controls, 13 PsA patients and 15 RA patients were analyzed. Almost all B cells expressed CXCR4 (91% of $CD19^+$ B cells) and there was no significant difference in numbers of $CXCR4^+$ B cells between PsA and RA patients and healthy controls ($p = 0.5895$). When compared to the expression of other chemokine receptors, B cells from all groups expressed higher levels of CXCR4 protein. There was no significant difference in mean fluorescence intensity between the three groups ($p = 0.1069$). However, there was a trend for PsA being the highest expressing group with mean fluorescence intensity of 343.4 ±23.1 SEM compared to healthy controls (256.8 ±9.7 SEM) and RA patients (284.5 ±15.8 SEM). The highest variation was seen in the PsA group with a range in mean fluorescence intensity from 210 to 483.

A


Counts
400
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B

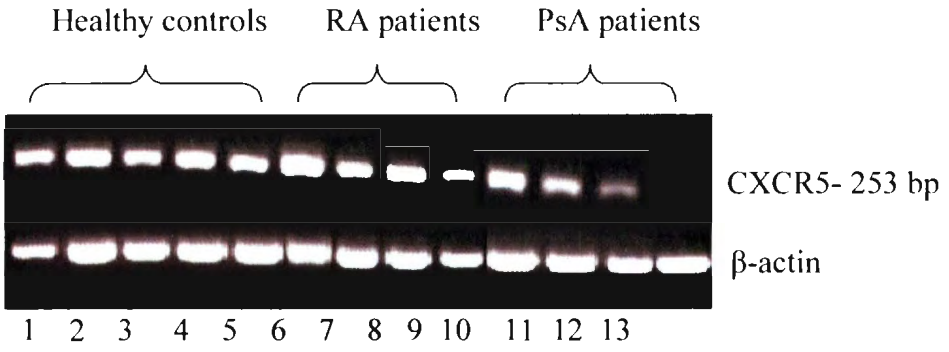

positive B cells [%]
100
90
80
70
60
mean fluorescence [MFI]
600
500
400
300
200
100
0
HC
RA
PsA
HC
RA
PsA

3.2.5 CXCR5 expression

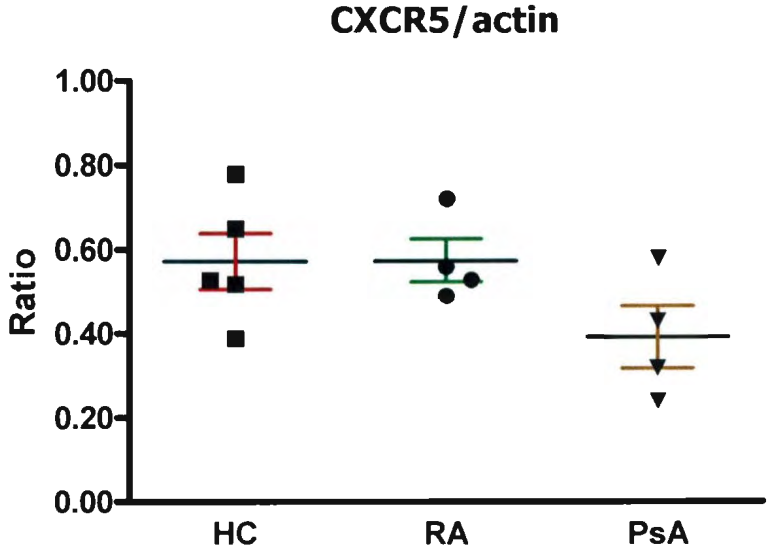
a) RT-PCR

Five healthy controls, four RA patients and four PsA patients were used for RT-PCR analysis to determine CXCR5 mRNA expression. The net intensities of the CXCR5 bands were normalized against the corresponding β-actin bands (ratio of CXCR5/β-actin). As shown in Fig. 3.5, all groups expressed medium amounts of CXCR5 mRNA (ratio for healthy control and RA patients 0.574 ±0.066 SEM and 0.575 ±0.050 SEM respectively, ratio for PsA patients 0.393 ±0.074 SEM) with no statistically significant difference between the groups ($p = 0.2346$). The expressed amount of CXCR5 mRNA was moderate and similar among the majority of samples so no further samples were analyzed for CXCR5 mRNA expression.

A


Healthy controls
RA patients
PsA patients
CXCR5- 253 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13

B

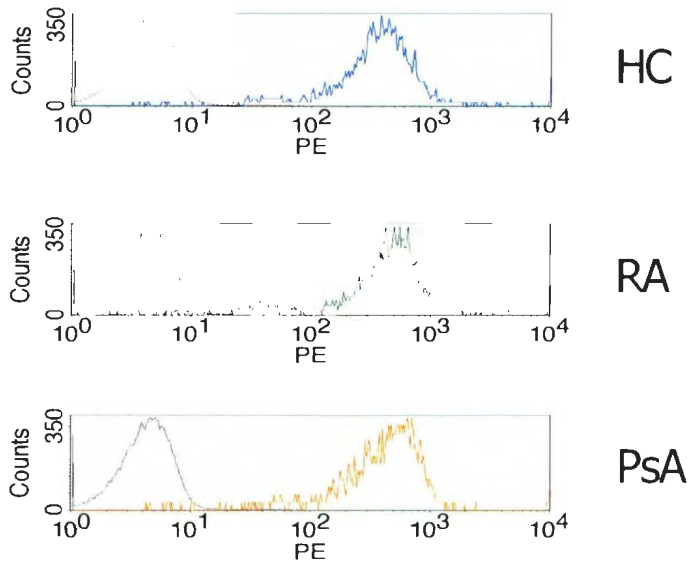

CXCR5/actin
Ratio
1.00
0.80
0.60
0.40
0.20
0.00
HC
RA
PsA

b) Flow cytometry

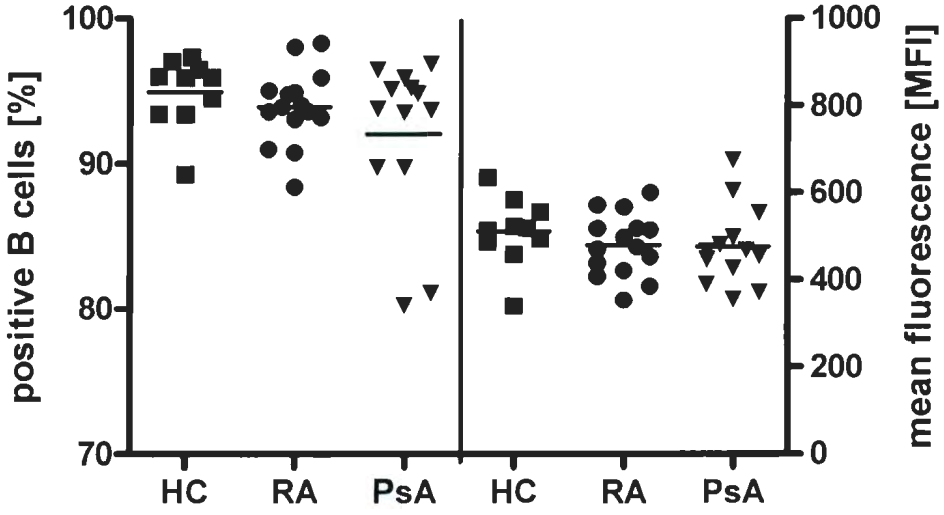
The results of CXCR5 expression analysis at the protein level are shown in Fig. 3.6. Samples from ten healthy controls, 13 PsA patients and 15 RA patients were analyzed. B cells from all three groups expressed CXCR5 (92-95% of $CD19^+$ B cells) and no statistically significant difference was seen between the three investigated groups ($p = 0.3059$). The highest variation was seen in the PsA group with a range of 80.3% to 96.9 % B cells expressing CXCR5, which was due to two samples that appear to be outliers. Excluding these two samples did not result in a significant difference in $CXCR5^+$ B cell numbers between the three groups ($p = 0.3972$).

In comparison to the other investigated chemokine receptors, CXCR5 showed the highest expression level by B cells from all three groups. There was no significant difference in mean fluorescence intensity between the three groups ($p = 0.3254$) and the variance within each group was similar for all groups (mean fluorescence intensity for PsA patients 476.3 ±25.12 SEM, for RA patients 479.5 ±18.38 SEM and healthy controls 511.5 ±24.86 SEM).

A


Counts
350
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B


positive B cells [%]
100
90
80
70
mean fluorescence [MFI]
1000
800
600
400
200
0
HC
RA
PsA
HC
RA
PsA

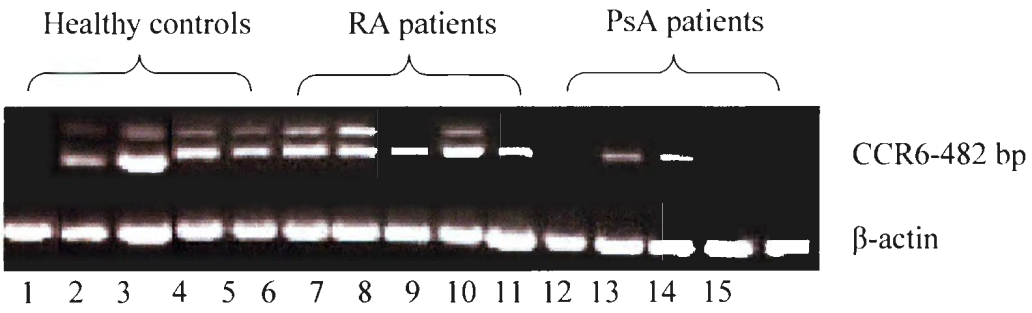
3.2.6 CCR6 expression

a) RT-PCR

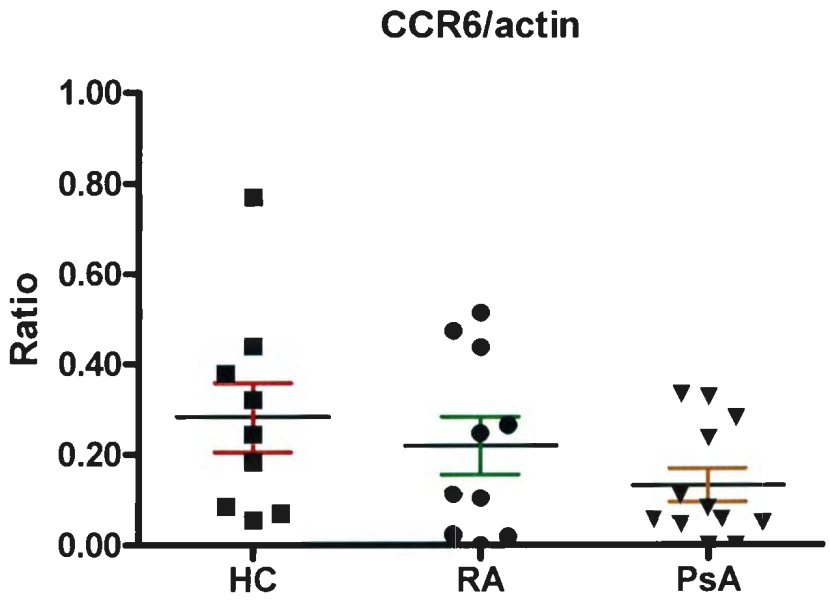
Nine healthy controls, ten RA patients and 12 PsA patients were analyzed using RT-PCR to determine CCR6 mRNA expression. The net intensities of the CCR6 bands were normalized against the corresponding β-actin bands (ratio of CCR6/β-actin).

All groups expressed medium amounts of CCR6 mRNA (mean ratio for healthy controls 0.284 ±0.076 SEM, mean ratio for RA patients 0.220 ±0.063 SEM and mean ratio for PsA patients 0.131 ±0.036 SEM) with no statistically significant difference ($p = 0.2066$) between the groups as shown in Fig. 3.7. The PsA group showed the lowest expression compared to the other groups. In all three groups the variance was high and ranged from not detectable to a ratio of 0.333 for PsA patients, not detectable to 0.514 for RA patients and from a ratio of 0.056 to 0.771 for healthy controls.

A


Healthy controls
RA patients
PsA patients
CCR6-482 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B


CCR6/actin
Ratio
1.00
0.80
0.60
0.40
0.20
0.00
HC
RA
PsA

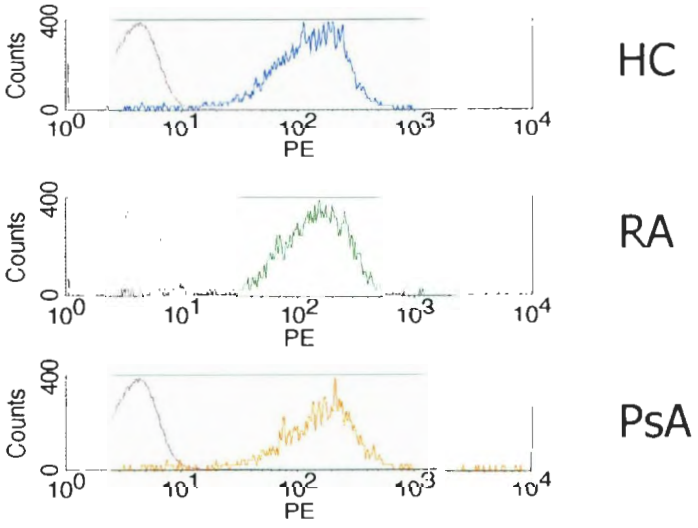
b) Flow cytometry

Fig. 3.8 shows the results of the flow cytometry experiment for ten healthy controls, 13 PsA patients and 15 RA patients that were used for analysis.

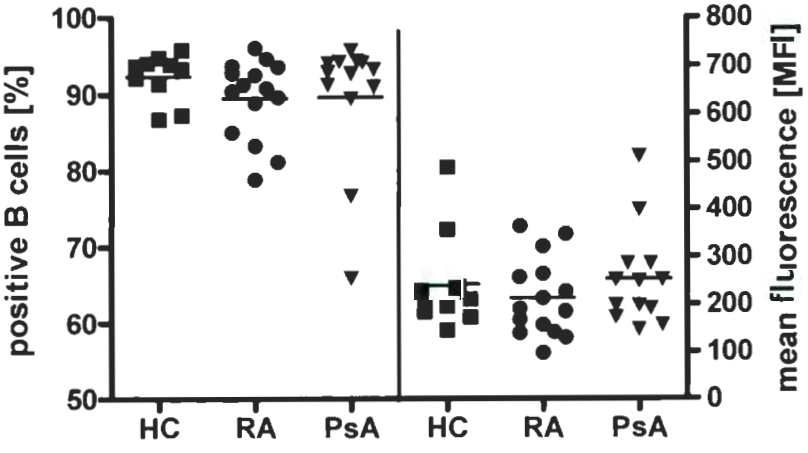
In all three groups the majority of B cells expressed CCR6 and no significant differences in numbers of $CCR6^+$ B cells were seen between any of the groups ($p = 0.2839$). The highest variance was seen in the PsA group with a mean of 89.69 ±2.39 SEM compared to the RA group (mean 89.52 ±1.33 SEM) and healthy controls (mean 92.34 ±0.96 SEM). This variance in the PsA group was due to two samples that appear to be outliers. Excluding these two samples did not result in a significant difference in $CCR6^+$ B cell numbers between the three groups ($p = 0.1063$).

In comparison to the other investigated chemokine receptors, CCR6 showed a medium level of expression by B cells from all groups. There was no significant difference ($p = 0.4824$) in mean fluorescence intensity between PsA patients (252.8 ±28.54 SEM), RA patients (213.2 ±21.22 SEM) and healthy controls (242.6 ±32.65 SEM). All three groups showed similar variance, for healthy controls and the PsA group this was mainly due to two samples that can be seen as outliers. Again, excluding these samples from the analysis did not result in a significant difference in CCR6 expression between the three investigated groups ($p = 0.7880$).

A


Counts
400
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B


positive B cells [%]
100
90
80
70
60
50
mean fluorescence [MFI]
800
700
600
500
400
300
200
100
0
HC
RA
PsA
HC
RA
PsA

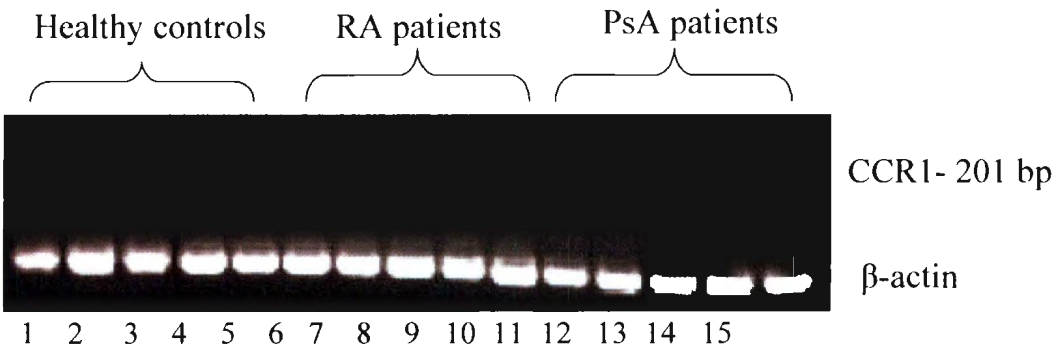
3.2.7 CCR1 expression

a) RT-PCR

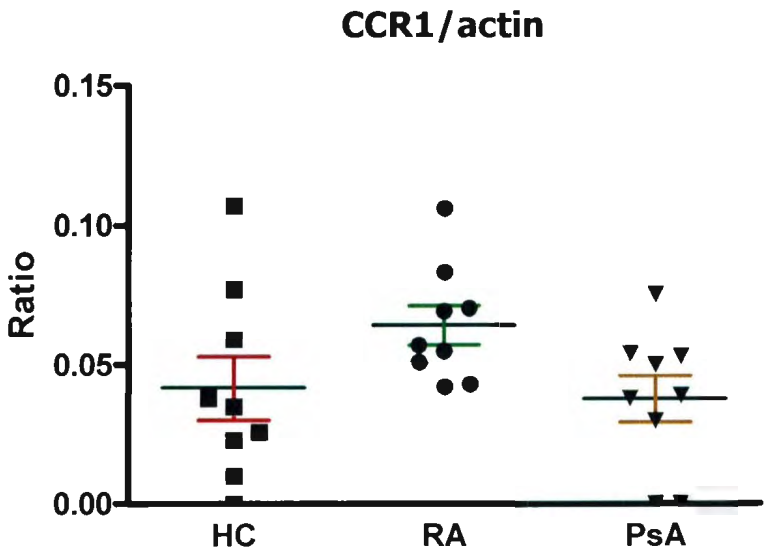
To determine the CCR1 mRNA expression using RT-PCR, nine samples per investigated group were analyzed. Again the CCR1 band intensities were normalized against the corresponding β-actin band intensities.

The results in Fig. 3.9 show low expression of CCR1 mRNA by PsA and RA patients as well as healthy controls. There was no significant difference in expression between the three groups ($p = 0.0679$) although there was a trend of RA being the highest expressing group with a mean ratio of 0.064 ±0.007 SEM compared to healthy controls (mean ratio 0.042 ±0.011 SEM) and PsA patients (mean ratio 0.038 ±0.008 SEM). The healthy control group had the highest variation with the CCR1 mRNA expression ranging from not detectable (ratio of 0) to a ratio of 0.107. Variation was also seen within the PsA group with two samples whose CCR1 mRNA expression could not be detected.

A


Healthy controls
RA patients
PsA patients
CCR1- 201 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

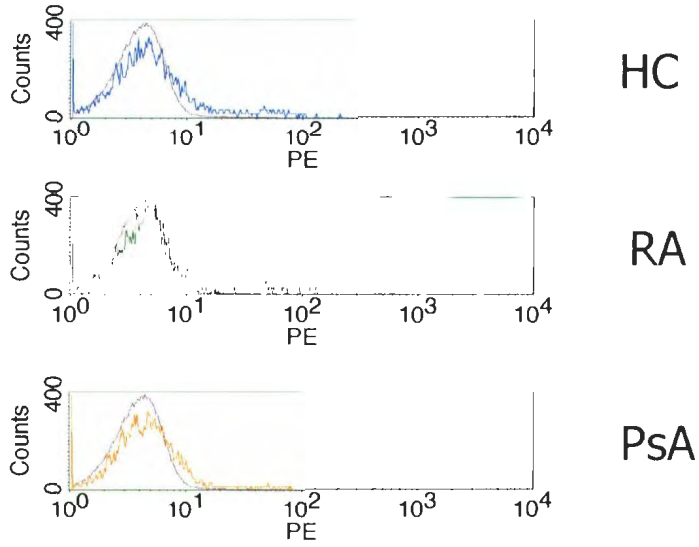
B


CCR1/actin
0.15
0.10
0.05
0.00
Ratio
HC
RA
PsA

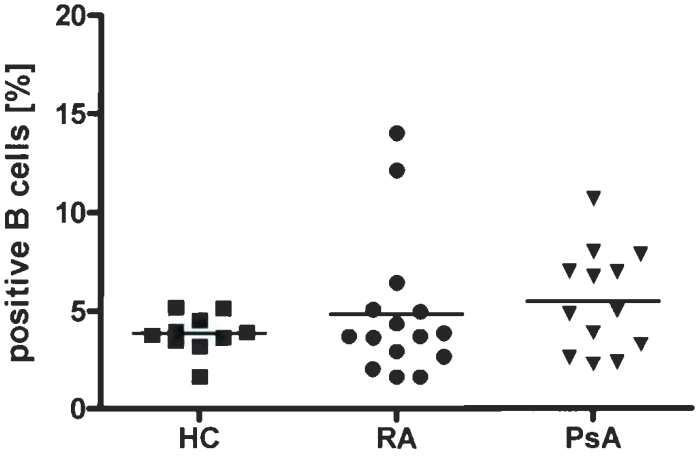
b) Flow cytometry

The results of the flow cytometry analysis for CCR1 are shown in Fig. 3.10. As for the other chemokine receptors, samples from ten healthy controls, 13 PsA patients and 15 RA patients were analyzed. The majority of $CD19^+$ B cells did not express measurable amounts of CCR1. For those with CCR1 expression, there was no statistically significant difference ($p = 0.3833$) in $CCR1^+$ B cell numbers between PsA patients (mean of 5.00% ±0.73 SEM), RA patients (mean of 4.84% ±0.93 SEM) and healthy controls (mean of 3.84% ±0.33 SEM). The highest variation in $CCR1^+$ B cell numbers within one group was observed for RA patients (range from 1.62% to 14.01%) and PsA patients (range from 2.22% to 10.69%). The variation in the RA patient group was due to two samples. Exclusion of these two samples from expression analysis did not result in a significant difference in $CCR1^+$ B cell numbers between the three groups ($p = 0.1290$) The amount of expressed CCR1 was low with no visible shift of the CCR1 peak compared to the isotype control for any of the analyzed groups. Thus the mean fluorescence intensity was not compared between the groups.

A


Counts
400
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B

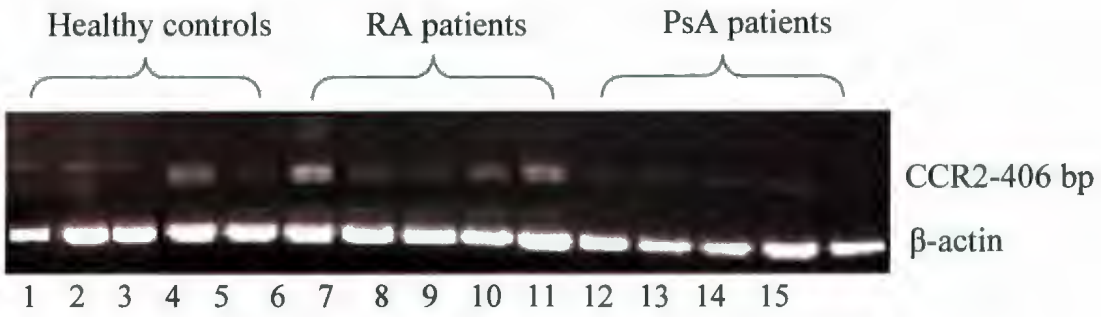

positive B cells [%]
20
15
10
5
0
HC
RA
PsA

3.2.8 CCR2 expression

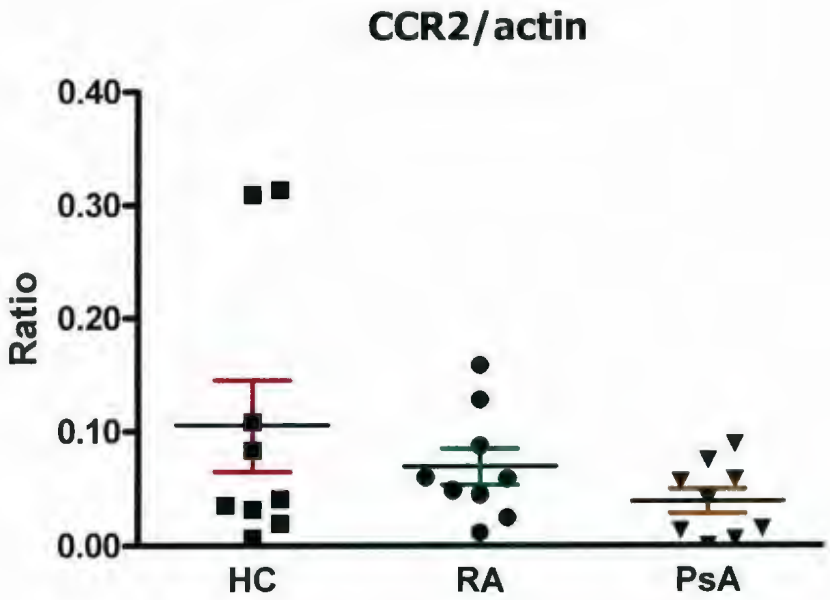
a) RT-PCR

Nine samples per group (healthy controls, PsA patients and RA patients) were used for RT-PCR analysis of CCR2 mRNA expression. As with the previously analyzed chemokine receptors, CCR2 bands were normalized against the corresponding β-actin band intensities. There was low expression of CCR2 mRNA by all groups as shown in Fig. 3.11. The PsA group was the lowest expressing group (0.039 ±0.011 SEM) compared to healthy controls (0.106 ±0.040 SEM) and RA patients (0.069 ±0.016 SEM), however the differences seen between the groups were not significant ($p = 0.3330$). The healthy control group showed the highest amount of variation with a range from 0.007 to 0.314. This was mainly due to two samples within this group that seem to be outliers. Excluding these samples from the analysis lowered the ratio for the healthy control group to 0.047 ±0.014 SEM but did not change the result of the statistical analysis ($p = 0.2975$).

A


Healthy controls
RA patients
PsA patients
CCR2-406 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B


CCR2/actin
Ratio
0.40
0.30
0.20
0.10
0.00
HC
RA
PsA

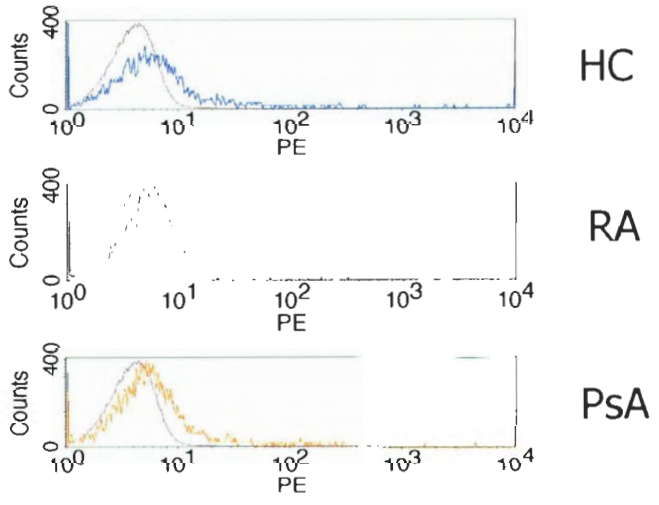
b) Flow cytometry

Ten healthy controls, 13 PsA patients and 15 RA patients were analyzed for CCR2 expression using flow cytometry as shown in Fig. 3.12.

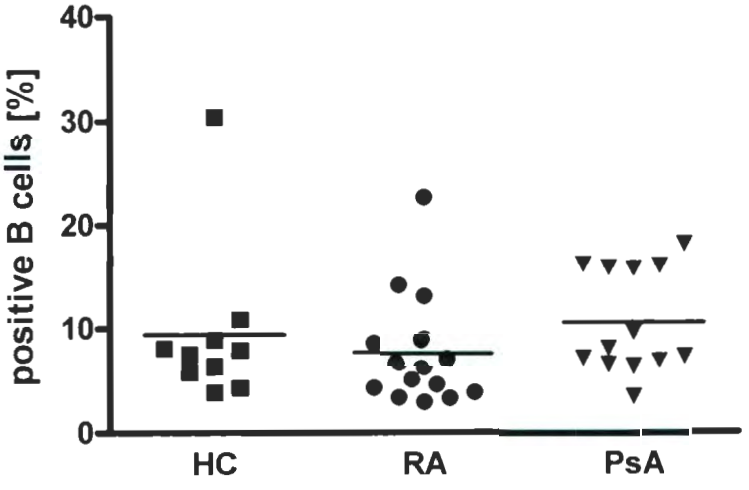
Only about 10% of the $CD19^+$ B cells actually expressed CCR2. No significant difference ($p = 0.1643$) in $CCR2^+$ B cell numbers was seen between PsA patients (mean 10.49% ± 1.39 SEM), RA patients (mean 7.69% ±1.39 SEM) and healthy controls (mean 9.41% ±2.43 SEM). The highest variation was in the patient groups (PsA range 3.37% – 18.09% and RA range 2.94 – 22.66%) and the variation seen for the healthy controls was mainly due to one sample. Excluding this sample did not result in a significant difference in $CCR2^+$ B cell numbers between the three groups ($p = 0.1401$).

The amount of expressed CCR2 was low with only a small shift of the CCR2 peak compared to the isotype control for any of the analyzed groups. Thus the mean fluorescence intensity was not compared between the groups.

A


Counts
400
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B


positive B cells [%]
40
30
20
10
0
HC
RA
PsA

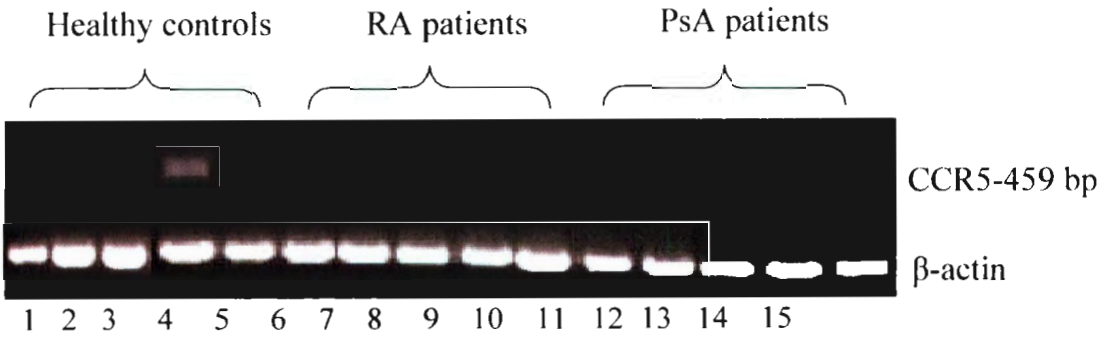
3.2.9 CCR5 expression

a) RT-PCR

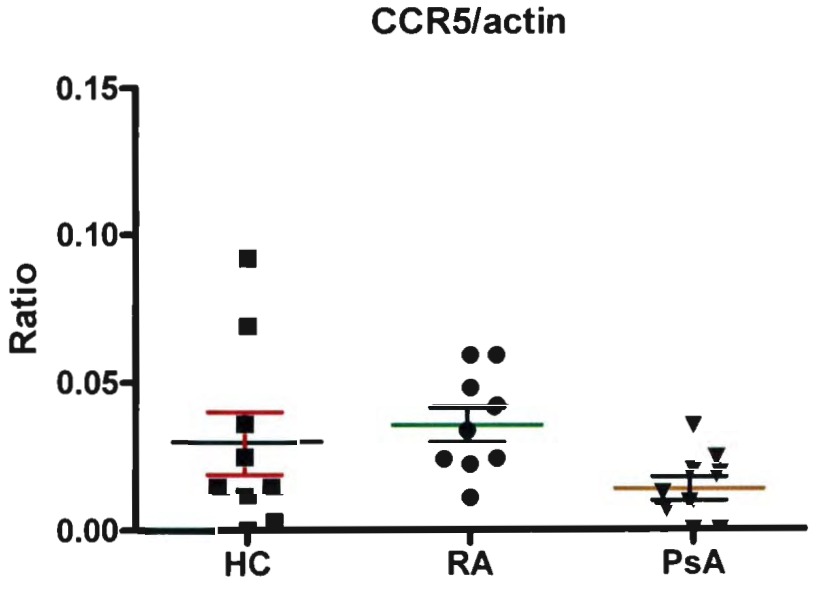
As for the previous receptors, nine samples per investigated group were used for CCR5 mRNA expression analysis. Again, CCR5 band intensities were normalized against the corresponding β-actin band intensities.

As shown in Fig. 3.13 all groups expressed very low amounts of CCR5 mRNA. There were no significant differences between the three analyzed groups ($p = 0.0656$), but the PsA group seemed to be the lowest expression group (ratio 0.014 ±0.004 SEM) compared to healthy controls (ratio 0.030 ±0.010 SEM) and RA patients (ratio 0.036 ±0.006 SEM). Some variance was seen in all three groups however the highest variance was seen for the healthy control group with sample ratios ranging from not detectable to 0.092. Again this was mainly due to two samples that appeared to be outliers. Excluding these outliers from the statistical analysis resulted in a significant difference between RA patients and PsA patients ($p = 0.0239$) with RA patients showing a higher expression compared to PsA patients.

A


Healthy controls
RA patients
PsA patients
CCR5-459 bp
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B


CCR5/actin
Ratio
0.15
0.10
0.05
0.00
HC
RA
PsA

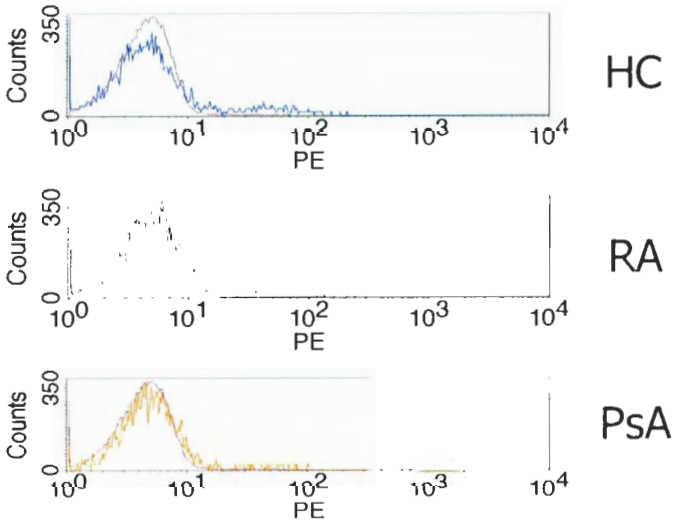
b) Flow cytometry

For CCR5 expression at the protein level, ten healthy controls, 13 PsA patients and 15 RA patients were analyzed using flow cytometry.

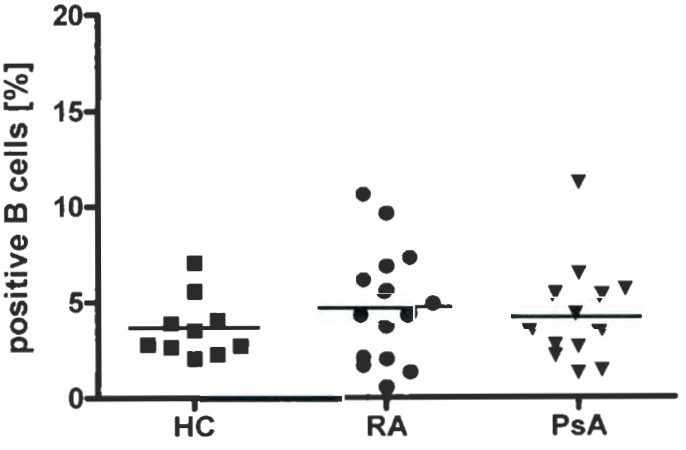
The results in Fig. 3.14 show that there was no significant difference of the mean number of B cells expressing CCR5 between the three groups ($p = 0.7864$). Numbers of cells expressing CCR5 was low, only about 4% of $CD19^+$ B cells expressed CCR5. For healthy controls it was 3.65% ±0.50 SEM, for PsA patients 4.29% ±0.74 SEM and for RA patients 4.75% ±0.78 SEM. The highest variation was seen in the RA group, ranging from 0.54% positive B cells to 10.65% positive B cells. The variation seen in the PsA group was mainly due to one sample that appears to be an outlier. Excluding this sample from the statistical analysis did not result in a significant difference in $CCR5^+$ B cell numbers between the investigated groups ($p = 0.6871$).

The amount of expressed CCR5 was low with no shift of the CCR2 peak compared to the isotype control for any of the analyzed groups. Thus the mean fluorescence intensity was not compared between the groups.

A


Counts
350
0
10^0
10^1
10^2
10^3
10^4
PE
HC
RA
PsA

B


positive B cells [%]
20
15
10
5
0
HC
RA
PsA

3.2.10 CD3 expression

Despite the high purity of the separated B cell population (94%-97% $CD20^+$ B cells) as determined by flow cytometry, expression of CD3 was analyzed using RT-PCR to exclude the influence of chemokine receptor-expressing T cells. The results are shown in Figure 3.15. All groups showed low expression of CD3 (mean ratio for healthy controls 0.116, mean ratio for RA patients 0.0852 and mean ratio for PsA patients 0.0547), which is similar to the ratio observed for low expressing chemokine receptors. This detection of CD3 expression was expected, as RT-PCR is sensitive enough to detect any T cells present in the enriched B cell population. The PsA group showed the lowest CD3 expression of the three groups, similarly the PsA group was observed to be the lowest expressing group for the chemokine receptors CXCR3, CCR2, CCR5 and CCR6, which are also expressed by T cells. To determine if this trend to be the lowest expressing group was due to less $CD3^+$ T cells present, possible correlation between the amount of T cell contamination (as determined by CD3 expression) and expression levels of the chemokine receptors were tested using the Spearman test. The results of this analysis are shown in Fig. 3.16. There were no statistically significant correlations observed between chemokine receptor expression and CD3 expression, but trends were visible. Healthy controls did show a trend for a positive correlation between CD3 expression and CXCR3 ($r = 0.90$, $p = 0.083$). PsA patients did show a trend for a positive correlation between CCR2 and CD3 expression ($r = 0.83$, $p = 0.058$). RA patients showed a negative trend for a correlation between CD3 and CCR2 expression ($r = -0.95$, $p = 0.083$) as well as a negative correlation between expression of CD3 and CCR6 ($r = -0.72$, $p = 0.136$).

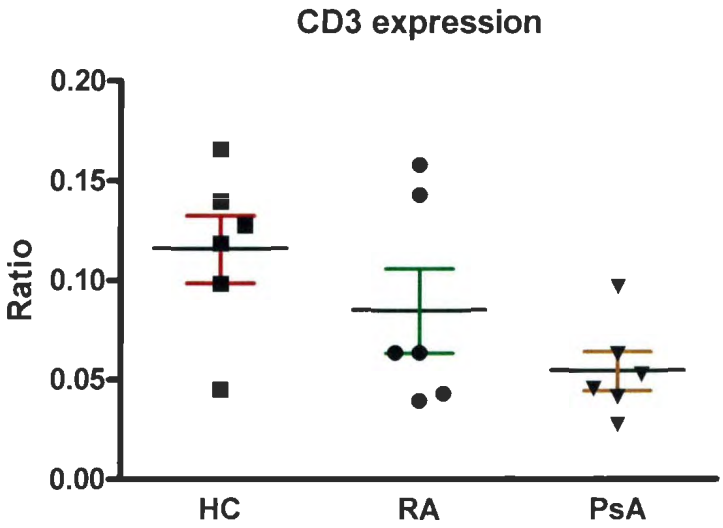
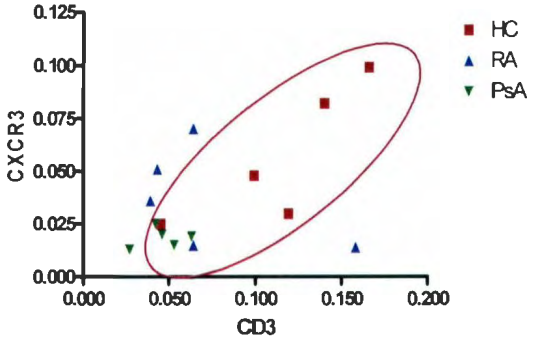


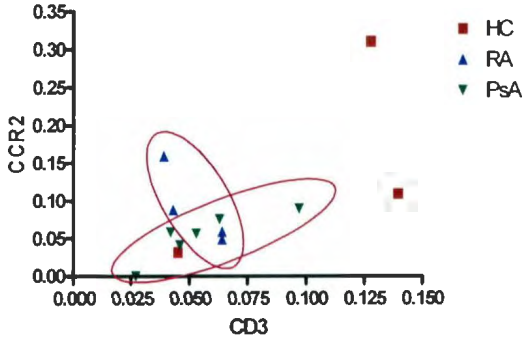
Figure 3.15: RT-PCR results for CD3 expression.

Scatter dot plot of band net intensities showing mean and standard error of mean in colour. The CD3 band net intensities were normalized against the corresponding β-actin band intensities (ratio = band intensity of CD3 band/band intensity of β-actin).

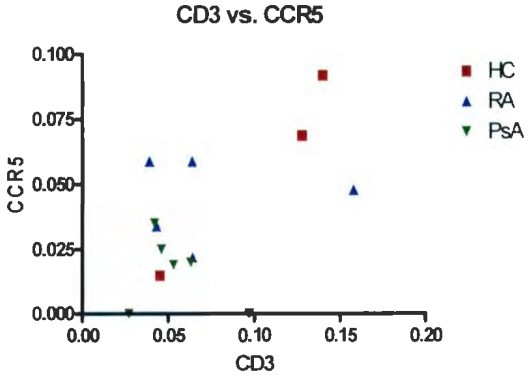
A


CD3 vs. CXCR3
CXCR3
CD3
0.125
0.100
0.075
0.050
0.025
0.000
0.000
0.050
0.100
0.150
0.200
HC
RA
PsA

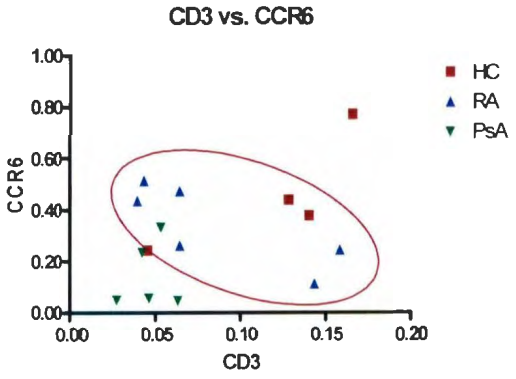
B


CD3 vs. CCR2
CCR2
CD3
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.000
0.025
0.050
0.075
0.100
0.125
0.150
HC
RA
PsA

C


CD3 vs. CCR5
CCR5
CD3
0.100
0.075
0.050
0.025
0.000
0.00
0.05
0.10
0.15
0.20
HC
RA
PsA

D


CD3 vs. CCR6
CCR6
CD3
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.05
0.10
0.15
0.20
HC
RA
PsA

3.3 Discussion

Differential expression of chemokine receptors promoting B cell migration to sites of inflammation in PsA and RA patients was investigated in this part of the thesis. To test this hypothesis, chemokine receptor expression was first analyzed at the RNA level using RT-PCR. The RT-PCR results are summarized in Table 3.2 (p. 61). These results showed that CXCR4 was expressed more strongly than any other chemokine receptor as determined by CXCR4 band intensity normalized against β-actin band intensity (CXCR4/β-actin ratios for all investigated groups >1). CXCR5 was also expressed strongly (ratios for all groups about 0.5). CCR6 showed a medium expression compared to other chemokine receptors. Lower expression levels were seen for CXCR3, CCR1, CCR2 and CCR5, with CCR5 showing the lowest expression.

To determine chemokine receptor expression at the protein level as well, the same receptors were also analyzed using flow cytometry. Firstly, the percentage of $CD19^+$ B cells expressing the analyzed chemokine receptors was investigated. The results of that experiment are summarized in Table 3.3 (p. 62) and show that the majority of $CD19^+$ B cells (>90%) expressed CXCR4, CXCR5 and CCR6. This was expected as these chemokine receptors have been reported to be constitutively expressed by B cells (David 2000). CXCR3 was expressed by about 75% of peripheral blood B cells whereas CCR1, CCR2 and CCR5 were only expressed by a fraction of B cells (<10%). These results for chemokine receptor expression on peripheral blood B cells are similar to previously published data (Henneken, Dorner et al. 2005) that show comparable percentages in

expression for healthy controls. However, in another publication CXCR3 was expressed by only 15%-25% of peripheral blood B cells (Jones, Benjamin et al. 2000).

The level of chemokine receptor expression was determined by measurement of mean fluorescence intensity and this is also summarized in Table 3.3. CXCR5 showed very high mean fluorescence intensity and was expressed the highest compared to the other chemokine receptors. CXCR4 and CCR6 also showed high expression whereas CXCR3 showed low expression. CCR1, CCR2 and CCR5 were expressed in such low amounts that it did not result in a measurable peak shift from the negative control. More B cells (8%-11%) expressed CCR2 than CCR1 (3%-6%) or CCR5 (3%-5%).

The comparison of the results obtained by RT-PCR and flow cytometry showed that the high expression for CXCR4 and CXCR5 seen at the protein level was also observed at the RNA level. However, CXCR4 showed stronger mRNA expression than CXCR5, which is probably due to contamination with other mononuclear cells (which were not measured), that also express CXCR4 (David 2000). CCR6 showed lower expression than CXCR4 and CXCR5 at the RNA level as well as the protein level. CXCR3 showed lower expression when analyzed using RT-PCR and although most B cells were actually positive for CXCR3 they appeared to express CXCR3 at a lower level compared to CXCR4, CXCR5 and CCR6 (between the three investigated groups of patients and controls mean fluorescence intensity ranged from 138.9-179.6 for CXCR3 compared to 278.8-343.4 for CXCR4 and 213.2-252.8 for CCR6). As seen at the protein level with more B cells expressing CCR2, the RT-PCR results showed that CCR2 was higher expressed with an average ratio of 0.071 compared to CCR1 and CCR5 expression

(average ratio of 0.048 and 0.027 respectively). Taken together the flow cytometry data supported and confirmed the results obtained with RT-PCR in terms of expression levels (as determined by mean fluorescence intensity) and the expression pattern of the chemokine receptors was similar as well.

We detected no differential expression between the three investigated groups (PsA patients, RA patients and healthy controls) for any of the analyzed chemokine receptors at the mRNA or the protein level. At the mRNA level, the PsA group showed a trend for being the lowest expressing group for CXCR3 and CCR5 ($p = 0.0598$ and $p = 0.0656$ respectively) whereas the RA group showed a trend to be the highest expressing group for CCR1 ($p = 0.0679$). At the protein level though the PsA group showed a trend to express higher levels of CXCR3 ($p = 0.0686$; mean fluorescence intensity of 179.6 compared to 143.1 for healthy controls and 138.9 for RA patients) and CXCR4 ($p = 0.1069$; mean fluorescence intensity of 343.4 compared to 278.8 for healthy controls and 284.5 for RA patients).

A possible influence of chemokine-receptor expressing T cells might explain the different trends seen at the mRNA and the protein level. To investigate this influence of T cells on the receptor expression seen at the mRNA level, CD3 expression was assessed using RT-PCR. All tested samples showed low CD3 expression as seen in Fig. 3.15, which was expected, as even a small $CD3^+$ T cell contamination would be enough to generate a PCR product. Interestingly, the T cell contamination varied between healthy controls, RA patients and PsA patients. The lowest amount of T cell contamination was seen for the PsA group, which also showed the lowest expression compared to the other groups for

CXCR3, CCR2, CCR5 and CCR6 expression. Possible correlation between CD3 expression (thus T cell contamination) and expression levels of those chemokine receptors was therefore analyzed using the Spearman test. The results are shown in Fig. 3.16. There were no significant correlations, but PsA patients and healthy controls both showed a trend towards positive correlation, while RA patients showed a trend towards negative correlation. This varying amount of T cell contamination in each group might also explain some of the differences in chemokine receptor expression seen between the groups. However, a positive correlation, which was expected since T cells do express all of the investigated chemokine receptors and the CD3 expression seemed to parallel the receptor expression, was not seen for the RA group. Also, CD3 expression data were not obtained for all the patient samples used for RT-PCR analysis of chemokine receptor expression therefore limiting the significance of the correlation data. Generally, the lack of significant differences in chemokine receptor expression might be explained by the high variance seen as it complicated analysis for both RT-PCR and flow cytometry. This was especially seen for receptors with low expression such as CCR1, CCR2 and CCR5. The RT-PCR results show that the healthy controls were the group with the highest variance for these low-expressing receptors. At the protein level, CCR1 and CCR5 showed variance in the number of receptor expressing B cells especially for PsA and RA patients. CCR6 showed variance for all groups for both the number of expressing B cells and the amount of expressed receptor. The amount of expressed CXCR3 varied the most for the PsA group. At the protein level, the variance seen for each group was often due to one or two samples that appeared to be outliers. This might

be indicative of subgroups within the healthy or patient population. The sample identification for each of these samples was researched to see if they would show a similar chemokine receptor expression pattern. Three PsA patients turned out to be responsible for most of the variation seen within the PsA group. However, these patients did not match in terms of chemokine receptor expression or clinical parameters such as disease severity, treatment, gender or age. The same was seen for the two RA patients identified to be responsible for most of the variance in the RA group.

The fact that the samples analyzed were from a heterogeneous pool of volunteers most likely accounts for the observed variance in expression, both at the RNA and the protein level. Similar individual variation was seen for flow cytometry data in a publication by Henneken et *al.* (Henneken, Dorner et al. 2005). To reduce variance and the possibility of type II error more samples would have to be analyzed. Likewise, calculation of the statistical power[15] for the trends seen at the protein level between healthy controls and PsA patients (using http://www.dssresearch.com/toolkit/default.asp) revealed a moderate power of 73.1% for CXCR4 and only 53% for CXCR3, consequently suggesting that more samples would be needed to detect a significant expression difference for both receptors. However, an increase in number of analyzed samples was not possible for all the chemokine receptors. This was due to limited blood sample quantities as the volume of blood available was restricted and some patients presented with a decreased number of PBMC. The sample volume was especially problematic for RT-PCR, because for this

[15] Statistical power is the ability of a statistical test to detect a difference, given that the difference actually exists

analysis the $CD19^+$ B cells were separated from the remaining PBMC and as B cells only comprise about 3-11% of the total PBMC population (K. Zipperlen, personal observation) RNA availability was limited.

Comparing the chemokine receptor expression only at the total B cell level might also explain the lack of differences in receptor expression seen between the three groups. Differences in expression of B cell subsets such as memory B cells might have been diluted out since the majority of peripheral blood B cells are recirculating naïve B cells.

Despite the lack of a significant differential expression of chemokine receptors on peripheral blood B cells in our study, an increase in inflammatory chemokine receptor expression on peripheral blood B cells would be expected in arthritis patients. This would allow B cells to migrate to the inflamed joint and would include CXCR3, CCR1, CCR2 and CCR5. These chemokine receptors are not normally constitutively expressed by B cells, but they are receptors for inflammatory chemokines (CXCL9 ligand for CXCR3, CCL5 ligand for CCR1 and CCR5, CCL2 ligand for CCR2) that are found in the synovial fluid of the inflamed joints (Konig, Krenn et al. 2000). The CCR6 ligand CCL20 could also serve as an attractant for B cells enhancing their migration to the skin as it is produced by keratinocytes in lesional psoriatic skin (Homey, Dieu-Nosjean et al. 2000). A homeostatic chemokine receptor like CXCR4 could also be expected to be up-regulated in expression as it has been shown on synovium infiltrating T cells. While not all seven investigated chemokine receptors are likely to show increased expression, we anticipated to see an increase for some that are important in B cell migration and that would be indicative of either a general phenomenon for arthritis patients or a specific

feature of PsA patients. This study could only show a trend for an increased expression of CXCR4 and CXCR3 for the PsA patient group compared to healthy controls and RA patients as determined by flow cytometry. Still, if significant this increased expression for CXCR4 and CXCR3 would show the involvement of these receptors in B cell migration specifically during PsA pathogenesis. A decreased expression on the other hand for some of the chemokine receptors could also be a possibility. This would be a reflection of the activation status of the B cell. A down-regulation of CCR6 is seen on activated B cells (Krzysiek, Lefevre et al. 2000) thus it may be possible to see this decreased expression of CCR6 on B cells from arthritis patients. This would then indicate the increased number of B cells with an activated phenotype. Similarly, decreased expression of CXCR5 is observed on activated B cells and after their differentiation into plasma cells (Henneken, Dorner et al. 2005) and would thus also be expected to be seen on B cells from arthritis patients.

Expecting differentially expressed chemokine receptors on lymphocytes migrating to specific tissue locations is in line with the endothelial area code model. This model implies that lymphocytes regulate their chemokine receptor and adhesion molecule profile after activation within secondary lymphoid organs to home to specific tissues (Kunkel, Boisvert et al. 2002; von Andrian and Mempel 2003; Burman, Haworth et al. 2005). A study by Burman et *al.* on the other hand suggests that the chemokine receptor expression gets modulated after lymphocytes enter the inflamed synovium (Burman, Haworth et al. 2005). Thus it seems possible that B cells change their chemokine receptor expression upon entry into the synovium and therefore it would be less likely to see

differences in expression on peripheral B cells. Similarly, activated B cells with an up-regulated inflammatory chemokine receptor profile might rapidly migrate to sites of inflammation therefore not contributing to inflammatory chemokine receptor expression of the total B cell population in the periphery.

Chemokine receptors are important for lymphocyte migration, which was shown here by the high expression of homeostatic chemokine receptors CXCR4, CXCR5 and CCR6. Also, CXCR3 is expressed by most B cells but in low amounts. However, there does not seem to be an involvement of differentially expressed chemokine receptors in recruiting peripheral blood B cells to the inflamed joints in patients with arthritis. Trends for expression differences were visible for some chemokine receptors but there was high individual variation within each group for many of the analyzed receptors thus complicating the comparison between PsA patients, RA patients and healthy controls. Furthermore, chemokine receptor expression did not correlate with disease status, treatment regiments or peripheral blood $CD19^+$ B cell numbers of individual patients. Henneken et *al.* previously showed up-regulation of CXCR3 and down-regulation of CXCR5 and CCR6 by peripheral blood B cells from RA patients, a finding which was not reproduced in this study. This difference in results can most likely be explained by the fact that a different group of patients and healthy volunteers was used in our study (therefore patients with different disease status, medication etc) compared to the study by Henneken et *al.*.

Future directions

Looking at B cell subsets for chemokine receptor expression would provide information on receptor expression on activated (memory B cells) and not activated cells (naïve B cells). This would potentially also aid in interpreting the outliers seen for the chemokine receptor expression at the protein level. Most likely activated B cells would show differential expression of chemokine receptors to migrate to sites of inflammation in contrast to re-circulating naïve B cells. However, for an optimal study to answer the question about the role of chemokine receptors in B cell migration, investigating infiltrating B cells in the inflamed joint in addition to peripheral blood B cells would be necessary. That way the expression profile of peripheral blood B cells could be put in context with the expression seen in the inflamed synovium. However, with necessary ethical regulations in place to protect the study participants, the accessibility to joint biopsies and joint fluid is restricted unless the patient would undergo the procedure for needed medical purposes.

CHAPTER 4

BAFF IN PSORIATIC ARTHRITIS

4.1 INTRODUCTION AND RATIONALE

The B cell activating factor BAFF is important for B cell activation and survival (see Introduction p. 32-34). About 20% of RA patients have elevated serum levels of BAFF compared to healthy controls (Cheema, Roschke et al. 2001). A recent presentation at the European Academy of Dermatology and Venereology meeting reported an increase in serum BAFF levels in psoriasis patients (JEADV 2007, 21, Suppl.1, 15-29). This is especially interesting, as psoriasis has so far been perceived as a T cell-mediated disease and not much focus has been placed on B cell involvement (Schon and Boehncke 2005). Different BAFF antagonists are currently under development and also being used in clinical trials for SLE and RA patients (Ramanujam and Davidson 2004). As BAFF can influence B cell function in many ways it seems likely that it contributes to an exaggerated B cell function in autoimmune diseases by activating and prolonging survival of B cells. Similarly, Lesley et *al.* concluded from the results of their study using monoclonal Ig-transgenic mice that under normal circumstances autoreactive B cells cannot compete for the limited amounts of BAFF present and are thus eliminated via BCR-induced death signals. They hypothesized that elevation of BAFF levels would lead to the prolonged survival of autoreactive B cells and therefore enhance pathogenic activity (Lesley, Xu et al. 2004).

BAFF levels also appear elevated in other autoimmune diseases with B cells contributing to disease pathology such as SLE and RA. It seems that elevated BAFF levels are indicative of a more prominent B cell involvement and help to sustain autoreactive B cell survival. Therefore, we hypothesized that if PsA had a B cell component in its

pathophysiology, patients may show elevated levels of BAFF. To test this hypothesis BAFF levels in plasma from PsA patients as well as RA patients and healthy controls were determined using ELISA.

4.2 Results

4.2.1 Plasma BAFF levels in PsA and RA patients

The plasma from 37 PsA patients, 24 RA patients and 35 healthy controls was used for BAFF determination by ELISA. As some of these samples were used for the previous chemokine study, the information on patients and healthy controls is summarized in Chapter 3, Table 3.1. As shown in Fig. 4.1 the mean BAFF levels were similar for the three groups with a mean of 970.5 ng/mL ±91.6 SEM for RA patients, 989.2 ng/mL ±47.7 SEM for PsA patients and 841.5 ng/mL ±67.4 SEM for healthy controls. There was no significant difference in BAFF levels between the three groups ($p = 0.1291$). BAFF levels higher than the mean plus two standard deviations of the healthy control samples were considered elevated. Only 2 RA patients had elevated BAFF levels whereas none of the PsA patients presented with elevated levels. High variation was seen for the RA group with values ranging from 346 ng/mL to 2223 ng/mL and the healthy control group (values from 231 ng/mL to 1958 ng/mL).

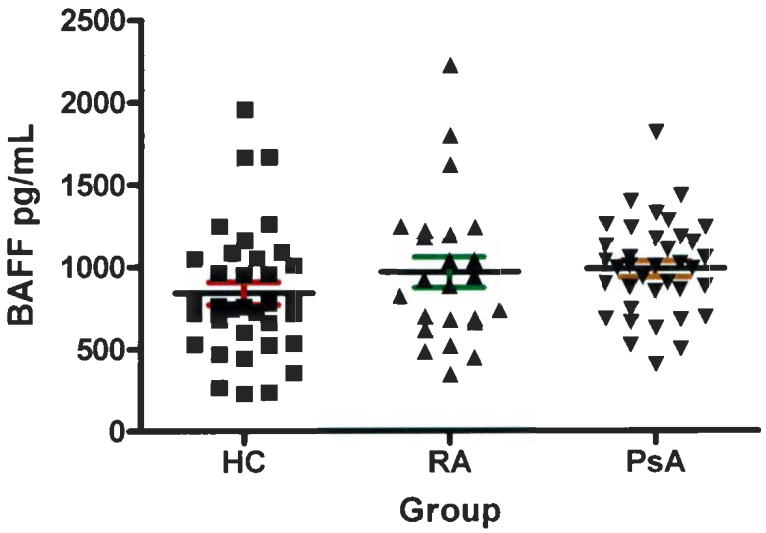


Figure 4.1: Plasma BAFF levels in healthy controls, PsA and RA patients.

This scatter dot plot shows the individual plasma BAFF amounts and the means plus SEM in colour for each analyzed group as determined by ELISA.

4.2.2 Correlation with clinical parameters and peripheral B cell numbers

To see whether disease status and treatment had an influence on BAFF levels, correlations of BAFF amounts with clinical parameters (joint count, ESR[16], RF[17] and treatment) were investigated. Information about joint counts, ESR and RF (IgM RF) was acquired from patient records. For 16 out of the 37 PsA patients ESR was measured the day the blood was taken. Treatment information was obtained from questionnaires filled in the same day. BAFF levels from RA and PsA patients were also correlated with numbers of peripheral $CD19^+$ B cells, which were determined using flow cytometry during the chemokine receptor analysis (double staining for chemokine receptor and CD19).

There appeared to be no correlation between BAFF amounts and disease severity as determined by joint counts, ESR and RF for both PsA and RA patients (data not shown). Analysis of the treatment data showed a positive association of RA patients receiving oral steroids and reduced BAFF levels compared to patients not receiving steroid treatment ($p = 0.0054$) as shown in Fig. 4.2 A. This was not seen for PsA patients (Fig. 4.2 B). Other treatments (methotrexate, anti-TNF agents) did not reveal associations with BAFF levels.

There was also no correlation between BAFF and the circulating levels of peripheral blood B cells for both RA and PsA patients (data not shown).

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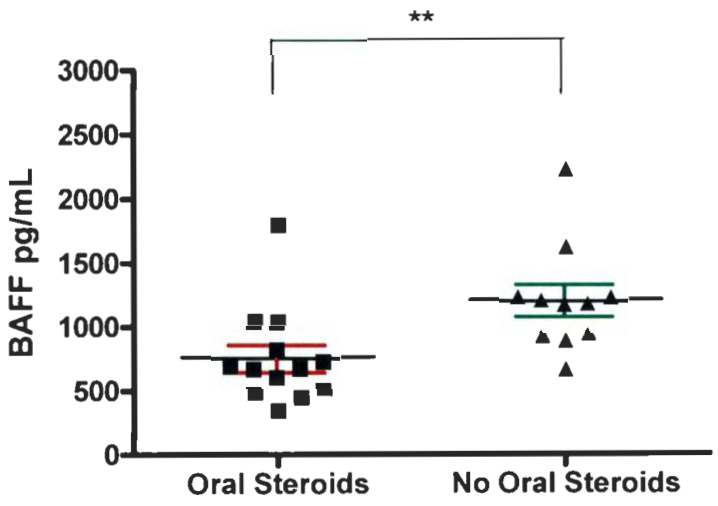
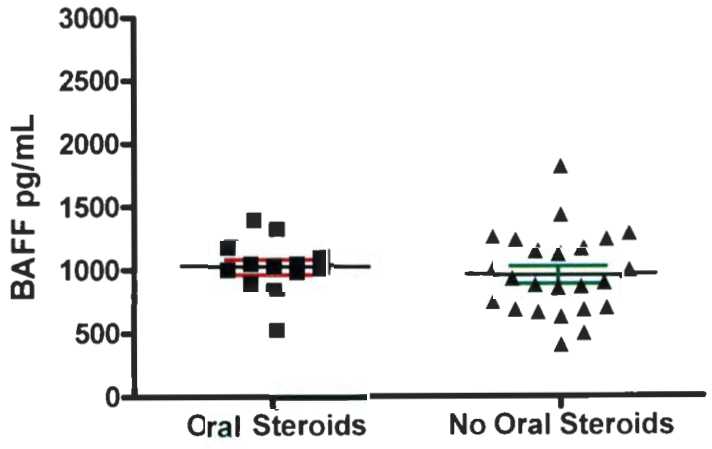
[16] ESR: Erythrocyte sedimentation rate

[17] only determined for RA patients

A


**
3000
2500
2000
1500
1000
500
0
BAFF pg/mL
Oral Steroids
No Oral Steroids

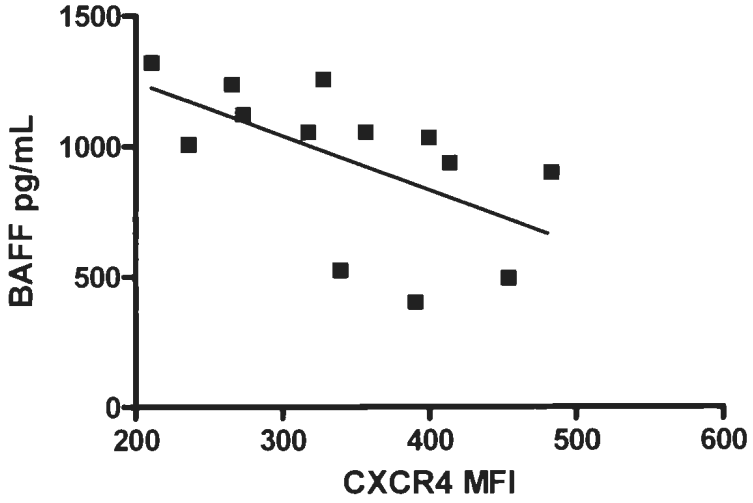
B


3000
2500
2000
1500
1000
500
0
BAFF pg/mL
Oral Steroids
No Oral Steroids

4.2.3 Comparison of BAFF and chemokine receptor expression levels

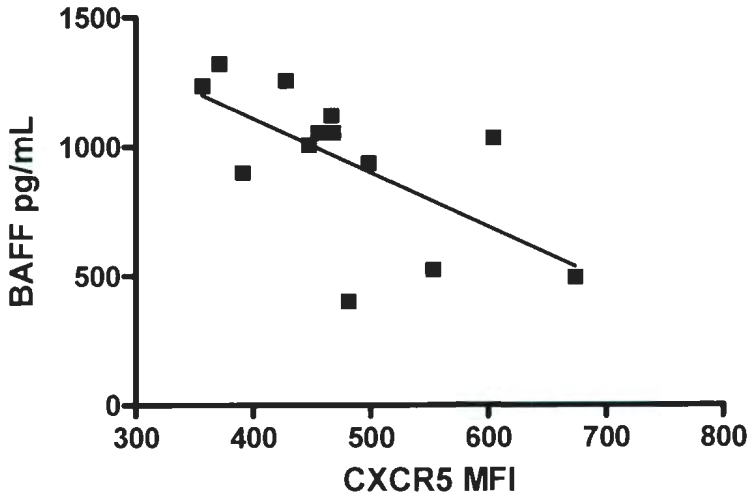
The BAFF levels were also correlated to the chemokine receptor expression of peripheral blood B cells, which was reported in Chapter 3. This was done for all three groups and the flow cytometry data were used for correlation analysis (percent positive B cells for CCR1, CCR and CCR5 and mean fluorescence intensity for CXCR3, CXCR4, CXCR5 and CCR6) using Spearman's test. The PsA group showed a significant inverse correlation between plasma BAFF levels and CXCR4 mean fluorescence intensity and CXCR5 mean fluorescence intensity ($r = -0.68$ with $p = 0.0103$ and $r = -0.66$ with $p = 0.0142$ respectively). The results of these correlations are shown in Fig. 4.3. Thus PsA patients with low expression of CXCR4 and CXCR5 on peripheral blood B cells have higher plasma BAFF levels. No significant correlation was found for the other chemokine receptors for the PsA group. Also, there were no significant correlations between BAFF levels and chemokine receptor expression for any of the studied chemokine receptors for the RA and the healthy control group.

A



r = -0.68, p = 0.0103

B



r = -0.66, p = 0.0142

4.3 Discussion

BAFF is an important survival factor for B cells and possibly plays a role in sustaining autoreactive B cells (Lesley, Xu et al. 2004). Increased BAFF has been found in the sera of patients with rheumatic autoimmune diseases such as SLE and RA (Cheema, Roschke et al. 2001). To see whether BAFF also contributes to autoimmune disease pathology in PsA, the plasma BAFF levels in PsA patients were determined using ELISA and compared to healthy controls as well as RA patients.

There was no significant increase in mean BAFF levels for PsA as well as RA patients compared to healthy controls. The heterogeneity in BAFF levels seen for all three groups in this study has been observed in other studies and has been attributed to individual variation of the recruited volunteers (Cheema, Roschke et al. 2001; Matsushita, Hasegawa et al. 2006). Also, a similar distribution of plasma BAFF values was seen for sample values provided by the supplier of the BAFF ELISA kit (R&D Systems) in the package insert, ranging from 609 pg/mL to 1946 pg/mL with a mean of 1000 pg/mL (http://www.rndsystems.com/pdf/dblys0.pdf). Another publication studying serum BAFF levels in systemic sclerosis noted a range of 390 to 1370 pg/mL serum BAFF for healthy participants (Matsushita, Hasegawa et al. 2006). Thus, the BAFF levels found for the healthy participants in our study (231 to 1985 ng/mL) seem to be in accordance with the above literature.

Only two RA patients (8%) had elevated BAFF levels, defining elevated as greater than two standard deviations from the mean BAFF level of the healthy controls, compared to no patients with elevated BAFF levels in the PsA group. This is in contrast to a study by

Cheema et *al.* that showed 20% of RA patients with elevated BAFF levels, defining elevated as greater than the 95th percentile seen for the healthy controls (Cheema, Roschke et al. 2001). This difference might be due to the use of different assay systems and individual variations of the different patient population.

The comparison of BAFF levels with peripheral blood B cell numbers did not reveal any correlation for RA as well as PsA patients. Similarly, a study by Cambridge et *al.* showed no correlation between BAFF levels and peripheral blood B cell numbers in RA patients (Cambridge, Stohl et al. 2006). Possibly BAFF levels only correlate with peripheral B cell numbers when there is a significant reduction of peripheral B cells due to disease or depletion therapy. An increase in BAFF levels would then indicate a compensation mechanism to replenish the depleted B cell population. Likewise, a strong inverse correlation between BAFF levels and peripheral B cell numbers was only observed in RA patients upon starting B cell depletion therapy (Cambridge, Stohl et al. 2006). Also, BAFF levels did not correlate with clinical parameters such as disease severity (as determined by joint counts), ESR and medications for PsA patients. Likewise there was no correlation of BAFF levels with joint counts, ESR or rheumatoid factor (IgM RF) for the RA patient group. The correlation of BAFF with rheumatoid factor has been controversial. A study by Cambridge et *al.* (Cambridge, Stohl et al. 2006) could not reproduce the correlation with RF reported in an earlier publication by Cheema et *al.* (Cheema, Roschke et al. 2001). The lack of correlation seen in our study is in line with the data from Cheema et *al.* as these authors did not find a correlation with IgM rheumatoid factor and BAFF levels (the highest correlation was found with IgG RF) and

the only information on RF available for this study was IgM rheumatoid factor (clinical testing for RF is performed on the IgM isotype). Generally, the correlation with clinical parameters (except patient treatment) was complicated as the information for most of the patients on joint counts, ESR and RF was accumulated retrospectively from hospital records and not assessed the day the sample was taken.

Correlating BAFF levels to patient treatment showed the influence of oral corticosteroids on BAFF levels in RA patients. There was a significant decrease in BAFF levels for RA patients receiving oral steroids compared to RA patients not receiving oral steroids. The patients receiving oral steroids were on low dose steroids (<10 mg/day). This reduction in BAFF levels was also observed by others studying Systemic Sclerosis patients receiving low dose steroids (5-20 mg/day) (Matsushita, Hasegawa et al. 2006). However, in a study involving SLE patients the influence of steroids on BAFF levels was only seen for high-dose steroids (>30 mg/day) (Stohl, Metyas et al. 2003; Matsushita, Hasegawa et al. 2006). This influence on BAFF levels by corticosteroid treatment was not seen in the PsA patient group despite PsA patients receiving the same or higher doses of steroids (however only 35% of PsA patients were on steroid treatment compared to 54% in the RA group). The effects of corticosteroids on molecular signalling pathways could possibly have a different impact in PsA patients compared to RA patients since PsA has a distinct disease pathogenesis. Stohl et *al.* hypothesized that BAFF levels in SLE patients receiving steroids are reduced because the steroids inhibit interferon (IFN), as IFN-γ and IFN-α usually up-regulate BAFF expression and are expressed at higher levels in SLE

patients (Stohl, Metyas et al. 2003). Thus they concluded that the steroid treatment in SLE leads to down-regulation of IFN and subsequently to the down-regulation of BAFF. Further comparison of BAFF levels to chemokine receptor expression on peripheral blood B cells revealed an inverse correlation between CXCR4 and CXCR5 expression and BAFF levels for the PsA group. No correlation was seen for the RA group or healthy controls for any of the studied chemokine receptors. So far it is not clear how plasma BAFF levels would affect the expression of chemokine receptors on B cells. High BAFF levels in patients with a decreased expression of CXCR4 and CXCR5 might be reflecting an increase of activated B cells within the total B cell pool. Henneken et *al.* investigated the downregulation of CXCR5 in their study and found that it occurs on activated B cells (Henneken, Dorner et al. 2005). However, information on CXCR4 and CXCR5 expression on peripheral blood B cells was not available for all the PsA patients with high plasma BAFF levels thus reducing the significance of this finding. Further research is warranted to investigate the importance of this correlation.

Future directions

Despite the lack of a significant difference in plasma BAFF levels between arthritis patients and healthy controls, it would be interesting to see whether BAFF levels correlate with the activation status of peripheral B cells. Generally comparing BAFF levels can be problematic as the BAFF levels found in plasma represent a balance between production of BAFF and consumption. Therefore measuring plasma BAFF levels might not be indicative of how much BAFF is actually 'seen' by B cells. To

address this issue the expression of BAFF-R or measuring BAFF RNA expression would be necessary. Also, more samples would need to be analyzed to assess the significance of the correlation between BAFF levels and chemokine receptor expression.

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APPENDIX

Flow cytometry data

CXCR3

Sample	Group	% positive cells	mean fluorescence intensity
KZ055	HC	72.19	160.13
KZ081	HC	81.05	125.37
KZ001	HC	85.25	163.86
KZ023	HC	77.29	172.65
KZ006	HC	73.49	117.62
KZ003	HC	71.62	108.23
KZ005	HC	86.06	156.61
KZ016	HC	78.54	183.49
KZ032	HC	80.26	141.81
KZ053	HC	68.95	100.74
KZ011	RA	61.19	151.60
KZ028	RA	69.31	140.18
KZ073	RA	82.97	188.37
KZ047	RA	89.84	94.71
KZ012	RA	71.43	159.56
KZ078	RA	74.36	124.06
KZ029	RA	65.14	121.68
KZ031	RA	61.88	128.21
KZ077	RA	83.23	147.87
KZ079	RA	87.02	88.28
KZ008	RA	72.66	163.91
KZ033	RA	57.11	143.26
KZ035	RA	57.85	130.38
KZ083	RA	63.23	157.49
KZ084	RA	57.95	143.44
KZ025	PsA	54.46	126.88
KZ067	PsA	75.40	187.43
KZ072	PsA	65.35	383.62
KZ030	PsA	81.43	180.24
KZ044	PsA	78.51	100.79
KZ076	PsA	72.78	173.91
KZ056	PsA	65.56	194.08
KZ010	PsA	87.13	152.66
KZ015	PsA	83.54	185.42
KZ068	PsA	79.08	160.23
KZ045	PsA	66.25	123.42
KZ050	PsA	41.31	150.43
KZ058	PsA	69.93	215.43

CXCR4

Sample	Group	% positive cells	mean fluorescence intensity
KZ055	HC	88.75	274.19
KZ081	HC	96.23	298.22
KZ001	HC	90.59	258.86
KZ023	HC	90.08	276.44
KZ006	HC	88.25	263.58
KZ003	HC	89.25	276.13
KZ005	HC	90.39	252.88
KZ016	HC	90.31	351.61
KZ032	HC	90.53	242.67
KZ053	HC	91.65	293.63
KZ011	RA	82.95	267.98
KZ028	RA	95.57	359.44
KZ073	RA	93.95	242.74
KZ047	RA	93.18	196.32
KZ012	RA	92.93	248.13
KZ078	RA	85.58	216.55
KZ029	RA	87.85	358.24
KZ031	RA	94.80	326.60
KZ077	RA	96.09	352.40
KZ079	RA	94.08	275.85
KZ008	RA	88.12	197.05
KZ033	RA	95.22	320.34
KZ035	RA	84.90	248.97
KZ083	RA	93.07	378.81
KZ084	RA	83.97	277.55
KZ025	PsA	90.59	390.11
KZ067	PsA	93.21	413.35
KZ072	PsA	84.72	235.89
KZ030	PsA	91.16	339.29
KZ044	PsA	94.75	317.26
KZ076	PsA	91.09	272.84
KZ056	PsA	92.49	399.30
KZ010	PsA	94.73	356.59
KZ015	PsA	96.82	453.67
KZ068	PsA	97.12	483.00
KZ045	PsA	92.64	327.55
KZ050	PsA	78.16	210.82
KZ058	PsA	86.03	264.92

CXCR5

Sample	Group	% positive cells	mean fluorescence intensity
KZ055	HC	89.26	340.36
KZ081	HC	97.33	556.41
KZ001	HC	97.05	459.65
KZ023	HC	96.52	635.33
KZ006	HC	93.39	487.65
KZ003	HC	96.02	523.14
KZ005	HC	95.94	513.78
KZ016	HC	93.40	519.67
KZ032	HC	95.97	494.95
KZ053	HC	94.49	584.02
KZ011	RA	93.16	600.32
KZ028	RA	93.58	438.51
KZ073	RA	95.93	353.97
KZ047	RA	94.95	385.08
KZ012	RA	98.00	475.00
KZ078	RA	93.89	497.74
KZ029	RA	88.39	518.05
KZ031	RA	93.55	421.66
KZ077	RA	98.29	567.72
KZ079	RA	95.01	517.58
KZ008	RA	93.03	571.58
KZ033	RA	94.03	514.28
KZ035	RA	91.01	407.95
KZ083	RA	94.78	470.51
KZ084	RA	90.77	452.63
KZ025	PsA	89.76	481.06
KZ067	PsA	95.22	498.29
KZ072	PsA	89.74	447.08
KZ030	PsA	93.79	552.71
KZ044	PsA	94.85	467.87
KZ076	PsA	96.50	466.15
KZ056	PsA	93.72	603.94
KZ010	PsA	95.92	455.20
KZ015	PsA	95.20	673.98
KZ068	PsA	96.88	390.64
KZ045	PsA	93.50	427.36
KZ050	PsA	81.11	370.73
KZ058	PsA	80.26	356.32

CCR1

Sample	Group	% positive cells
KZ055	HC	3.46
KZ081	HC	1.64
KZ001	HC	3.63
KZ023	HC	5.20
KZ006	HC	3.91
KZ003	HC	3.18
KZ005	HC	5.14
KZ016	HC	3.78
KZ032	HC	3.96
KZ053	HC	4.54
KZ011	RA	2.01
KZ028	RA	1.62
KZ073	RA	12.12
KZ047	RA	4.98
KZ012	RA	2.64
KZ078	RA	3.66
KZ029	RA	3.69
KZ031	RA	4.34
KZ077	RA	2.90
KZ079	RA	5.07
KZ008	RA	14.01
KZ033	RA	6.44
KZ035	RA	1.62
KZ083	RA	3.88
KZ084	RA	3.63
KZ025	PsA	2.35
KZ067	PsA	6.97
KZ072	PsA	7.89
KZ030	PsA	6.77
KZ044	PsA	2.58
KZ076	PsA	2.22
KZ056	PsA	7.00
KZ010	PsA	3.22
KZ015	PsA	8.01
KZ068	PsA	5.03
KZ045	PsA	3.88
KZ050	PsA	4.86
KZ058	PsA	10.69

CCR2

Sample	Group	% positive cells
KZ055	HC	4.35
KZ081	HC	3.88
KZ001	HC	7.50
KZ023	HC	10.87
KZ006	HC	8.08
KZ003	HC	8.88
KZ005	HC	30.44
KZ016	HC	6.39
KZ032	HC	7.92
KZ053	HC	5.80
KZ011	RA	3.36
KZ028	RA	3.29
KZ073	RA	22.66
KZ047	RA	7.20
KZ012	RA	14.17
KZ078	RA	5.13
KZ029	RA	6.33
KZ031	RA	6.75
KZ077	RA	2.94
KZ079	RA	8.58
KZ008	RA	13.11
KZ033	RA	8.95
KZ035	RA	4.63
KZ083	RA	3.88
KZ084	RA	4.30
KZ025	PsA	3.37
KZ067	PsA	16.10
KZ072	PsA	15.82
KZ030	PsA	15.67
KZ044	PsA	7.04
KZ076	PsA	6.83
KZ056	PsA	18.09
KZ010	PsA	7.24
KZ015	PsA	15.95
KZ068	PsA	9.51
KZ045	PsA	6.46
KZ050	PsA	6.33
KZ058	PsA	7.98

CCR5

Sample	Group	% positive cells
KZ055	HC	2.04
KZ081	HC	2.71
KZ001	HC	3.89
KZ023	HC	2.78
KZ006	HC	3.50
KZ003	HC	2.26
KZ005	HC	7.05
KZ016	HC	4.08
KZ032	HC	5.57
KZ053	HC	2.63
KZ011	RA	1.32
KZ028	RA	2.01
KZ073	RA	4.38
KZ047	RA	4.93
KZ012	RA	1.68
KZ078	RA	2.10
KZ029	RA	9.62
KZ031	RA	0.54
KZ077	RA	4.37
KZ079	RA	6.14
KZ008	RA	10.65
KZ033	RA	3.72
KZ035	RA	7.31
KZ083	RA	6.84
KZ084	RA	5.60
KZ025	PsA	1.40
KZ067	PsA	5.36
KZ072	PsA	5.64
KZ030	PsA	4.46
KZ044	PsA	2.62
KZ076	PsA	1.26
KZ056	PsA	2.15
KZ010	PsA	3.49
KZ015	PsA	5.42
KZ068	PsA	6.45
KZ045	PsA	2.71
KZ050	PsA	3.54
KZ058	PsA	11.22

CCR6

Sample	Group	% positive cells	mean fluorescence intensity
KZ055	HC	91.38	145.73
KZ081	HC	94.83	196.60
KZ001	HC	93.75	201.74
KZ023	HC	95.80	237.63
KZ006	HC	86.76	184.32
KZ003	HC	92.19	172.68
KZ005	HC	93.92	210.82
KZ016	HC	94.10	356.93
KZ032	HC	93.36	231.39
KZ053	HC	87.33	488.09
KZ011	RA	91.29	213.32
KZ028	RA	92.54	190.27
KZ073	RA	93.59	156.18
KZ047	RA	94.64	184.86
KZ012	RA	88.90	167.71
KZ078	RA	90.80	226.75
KZ029	RA	83.32	347.46
KZ031	RA	92.79	257.04
KZ077	RA	96.07	362.95
KZ079	RA	90.42	321.03
KZ008	RA	81.21	129.96
KZ033	RA	93.72	263.54
KZ035	RA	78.90	97.00
KZ083	RA	89.63	140.84
KZ084	RA	85.04	139.06
KZ025	PsA	91.01	251.21
KZ067	PsA	92.91	155.03
KZ072	PsA	91.28	172.65
KZ030	PsA	94.11	251.07
KZ044	PsA	92.74	284.98
KZ076	PsA	94.19	190.44
KZ056	PsA	94.39	284.65
KZ010	PsA	94.28	247.88
KZ015	PsA	95.75	511.04
KZ068	PsA	93.27	197.09
KZ045	PsA	89.47	197.73
KZ050	PsA	65.89	396.97
KZ058	PsA	76.68	145.64

RT-PCR data

CXCR3

Sample	Group	Ratio
KZ081	HC	0.082
KZ055	HC	0.025
KZ003	HC	0.048
KZ032	HC	0.099
KZ053	HC	0.030
KZ073	RA	0.036
KZ012	RA	0.070
KZ077	RA	0.015
KZ078	RA	0.051
KZ079	RA	0.014
KZ072	PsA	0.020
KZ076	PsA	0.025
KZ015	PsA	0.015
KZ067	PsA	0.019
KZ068	PsA	0.013

CXCR4

Sample	Group	Ratio
KZ013	HC	1.413
KZ004	HC	1.391
KZ026	HC	1.008
KZ001	HC	1.282
KZ006	HC	1.129
KZ028	RA	1.035
KZ031	RA	1.114
KZ047	RA	1.270
KZ011	RA	0.598
KZ029	RA	0.641
KZ030	PsA	1.003
KZ010	PsA	1.201
KZ044	PsA	1.139
KZ025	PsA	0.554
KZ056	PsA	1.697

CXCR5

Sample	Group	Ratio
KZ026	HC	0.780
KZ013	HC	0.530
KZ004	HC	0.650
KZ006	HC	0.520
KZ001	HC	0.390
KZ034	RA	0.720
KZ028	RA	0.490
KZ031	RA	0.560
KZ047	RA	0.530
KZ030	PsA	0.580
KZ010	PsA	0.430
KZ044	PsA	0.320
KZ056	PsA	0.240

CCR1

Sample	Group	Ratio
KZ026	HC	0.010
KZ013	HC	0.000
KZ006	HC	0.035
KZ001	HC	0.026
KZ081	HC	0.107
KZ055	HC	0.059
KZ016	HC	0.023
KZ032	HC	0.038
KZ053	HC	0.077
KZ072	PsA	0.054
KZ076	PsA	0.038
KZ015	PsA	0.000
KZ067	PsA	0.075
KZ068	PsA	0.030
KZ030	PsA	0.039
KZ010	PsA	0.050
KZ044	PsA	0.053
KZ056	PsA	0.000
KZ035	RA	0.057
KZ028	RA	0.043
KZ031	RA	0.051
KZ047	RA	0.055
KZ073	RA	0.106
KZ012	RA	0.083
KZ077	RA	0.069
KZ078	RA	0.042
KZ079	RA	0.070

CCR2

Sample	Group	Ratio
KZ026	HC	0.041
KZ013	HC	0.007
KZ023	HC	0.084
KZ006	HC	0.036
KZ001	HC	0.314
KZ004	HC	0.020
KZ081	HC	0.109
KZ055	HC	0.032
KZ016	HC	0.310
KZ029	RA	0.012
KZ028	RA	0.045
KZ031	RA	0.060
KZ047	RA	0.025
KZ073	RA	0.159
KZ012	RA	0.059
KZ077	RA	0.049
KZ078	RA	0.088
KZ079	RA	0.128
KZ030	PsA	0.015
KZ010	PsA	0.013
KZ044	PsA	0.006
KZ072	PsA	0.041
KZ076	PsA	0.058
KZ015	PsA	0.056
KZ067	PsA	0.075
KZ068	PsA	0.000
KZ037	PsA	0.089

CCR5

Sample	Group	Ratio
KZ026	HC	0.036
KZ013	HC	0.015
KZ023	HC	0.025
KZ006	HC	0.012
KZ001	HC	0.000
KZ004	HC	0.003
KZ081	HC	0.092
KZ055	HC	0.015
KZ016	HC	0.069
KZ029	RA	0.011
KZ028	RA	0.024
KZ031	RA	0.042
KZ047	RA	0.024
KZ073	RA	0.059
KZ012	RA	0.059
KZ077	RA	0.022
KZ078	RA	0.034
KZ079	RA	0.048
KZ030	PsA	0.013
KZ010	PsA	0.010
KZ044	PsA	0.007
KZ072	PsA	0.025
KZ076	PsA	0.035
KZ015	PsA	0.019
KZ067	PsA	0.020
KZ068	PsA	0.000
KZ037	PsA	0.000

CCR6

Sample	Group	Ratio
KZ001	HC	0.070
KZ013	HC	0.184
KZ004	HC	0.056
KZ023	HC	0.321
KZ006	HC	0.086
KZ081	HC	0.379
KZ055	HC	0.245
KZ016	HC	0.440
KZ032	HC	0.771
KZ011	RA	0.020
KZ029	RA	0.023
KZ028	RA	0.105
KZ073	RA	0.436
KZ012	RA	0.473
KZ077	RA	0.264
KZ078	RA	0.514
KZ079	RA	0.246
KZ056	PsA	0.000
KZ025	PsA	0.056
KZ030	PsA	0.110
KZ010	PsA	0.281
KZ072	PsA	0.057
KZ076	PsA	0.236
KZ015	PsA	0.333
KZ067	PsA	0.046
KZ068	PsA	0.049
KZ035	PsA	0.113